REMARKS

I. Introduction

Applicants have amended the priority claim to correct a typographical error, as suggested by the Examiner. Claim 10 has been deleted and new claims 11-17 have been added. Support for new claim 11 may be found, for example, at page 3, lines 5-9, and Example 8 (which demonstrates the anti-Influenza virus activity of the claimed hMBP with its unique domain structure comprising the extremely short collagen region, neck region, and carbohydrate recognition region) of the application as filed. Support for new claim 12 can be found in claim 10 as previously presented. Support for new claims 13-17 can be found, for example, in Example 1 of the application as field.

II. The objection to the specification may be withdrawn.

The Examiner objected to the specification for the recitation of an incorrect application number. Applicant submits that the aforementioned formality has been corrected. Accordingly, the Examiner's objection to the specification may be withdrawn.

III. The rejection of claim 10 under 35 U.S.C. § 112, first paragraph, may be withdrawn.

The Examiner rejected claim 10 for assertedly failing to comply with the written description requirement. Specifically, the Examiner asserted that claim 10 recites the term "isolated" which is not supported by the specification. The rejection is erroneous because a person of ordinary skill in the field would understand that the inventor contemplated "isolated" hMBP. Whether or not the application (as translated) uses the word "isolated" is not dispositive. However, the term is not necessary to describe the invention. Claim 10 has been canceled, and new claims 11-17 do not recite the term "isolated." Accordingly, Applicant submits that the rejection of claim 10 under 35 U.S.C. § 112, first paragraph, has been overcome and should be withdrawn.

IV. The rejection of claims 10 and under 35 U.S.C. § 112, second paragraph, may be withdrawn.

The Examiner rejected claim 10 under 35 U.S.C. § 112, second paragraph, for assertedly failing to particularly point out and distinctly claim the subject matter which the Applicant regards as the invention. In response to the Examiner's request, Applicant submits

herewith a copy of the Malhotra paper (Exhibit A). As previously suggested, Malhotra *et al.*, teach that the collagen-like region and ability to bind carbohydrates are general characteristics of collectins (See, e.g., page 1444, bottom of left column and bridging right column). Furthermore, the phrase "N terminal region carrying cysteine," which the Examiner asserted was unclear, is not recited in new claims 11-17.

In the Office action sent on September 17, 2003, the Examiner asserted that "The metes and bounds of "an N-terminal region containing a cysteine, a collagen-like region, a neck region and a carbohydrate recognition domain" are not known." Applicant submits that these terms of the art with known meaning (See, for example, Figure 1A and 1B, and page 1143, right column, line 16, to page 1144, left column, line 14 of Hoppe et al., attached hereto as Exhibit B)

Accordingly, Applicants submit that the rejection of claim 10 under 35 U.S.C. § 112, second paragraph should be withdrawn.

V. The rejection of claims 10 and under 35 U.S.C. § 102(b), may be withdrawn.

The Examiner rejected claim 10 under 35 U.S.C. § 102(b) as being anticipated by Kawasaki *et al.* The Examiner asserted that Kawasaki *et al.* teach an isolated hMBP and that the hMBP of Kawasaki *et al.* and the present invention are the same, regardless of the claimed method steps of the invention.

In response, Applicant first points out that claim 10 has been canceled and new claims 11-17 have been added. Applicant further submits that Kawasaki *et al.* do not disclose the hMBP of the present invention. Kawasaki *et al.* merely disclose the isolated serum lectin (i.e., the natural product) from human serum specific for mannose and N-acetylglucosamine (See Abstract; page 937, right column, lines 9-11). Kawasaki *et al.* do not disclose a genetically-altered serum lectin.

In contrast, the pending claims of the instant invention describe a hMBP that has a unique domain structure in comparison with the known, natural MBP domain structure. Specifically, the hMBP of the instant invention has been genetically engineered to include only six amino acid residues of a collagen-like region (i.e., only 2 units of Gly-Xaa-Xaa), a neck region, and a carbohydrate recognition domain (CRD). Additional Gly-Xaa-Xaa units

are present in the native protein of Kawasaki et al. Moreover, claim 11 does not recite an N-terminal region carrying cysteine, even though such a domain is present in the native protein.

For the foregoing reasons, Applicant submits that the rejection of claim 10 under 35 U.S.C. § 102, has been overcome and should be withdrawn.

CONCLUSION

In view of the amendments and remarks made herein, Applicants submit that claims 11-17 are in condition for allowance and request notification of the same.

Dated: November 12, 2004

Respectfully submitted,

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Interaction of C1q receptor with lung surfactant protein A*

Earlier we reported the purification of C1q receptor (C1qR) from U937 cells and human tonsil lymphocytes (Malhotra, R. and Sim, R. B., *Biochem. J.* 1989. *218:* 625) and showed that C1qR interacts with the ligands C1q, mannose-binding protein, conglutinin and lung surfactant protein A (SP-A) (Malhotra, R., Thiel, S., Reid, K. B. M. and Sim, R. B., *J. Exp. Med.* 1990. *172:* 955). C1qR was characterized as an acidic glycoprotein, which, when solubilized, exists as a dimer of M_r 115 000 under non-denaturing conditions. In this article we provide evidence for binding of radioiodinated SP-A to U937 cells and show that binding of radioiodinated SP-A to U937 cells is specific, saturable, salt dependent and is inhibited by purified C1qR and by C1q. The interaction of SP-A with U937 cells was found to up-regulate the surface expression of C1qR. Incubation of SP-A with U937 cells at 37 °C for 80 min was found to increase the receptor number per cell. Increase in receptor number was inhibited in the presence of sodium azide and monensin. Incubation of cells with calcium ionophore A 23187 induced increased surface expression in the absence of SP-A. The results indicate that interaction of SP-A with U937 cells triggers the expression of an intracellular pool of C1qR.

1 Introduction

Lung surfactant is secreted into the alveoli by alveolar type-II cells and bronchoalveolar cells. The lung surfactant is composed of phospholipids, cholesterol, other neutral lipids and proteins [1]. The surfactant is morphologically and functionally heterogeneous and can be separated by sequential centrifugation into two structural forms, termed multilayer vesicles and tubular myelin or small vesicles. Multilayer vesicles and tubular myelin lower the surface tension more effectively when they are associated with proteins [2, 3]. Three of the major protein components which have been characterized are termed SP-A, SP-B and SP-C [4], and a recently described further component is SP-D [5]. SP-A is the most abundant protein, with a concentration of 3 mg per 100 mg of phospholipids. It is a complex molecule composed of 18 polypeptide chains, each 228 amino acids long. These associate to form collagen triple helices and globular domains. The ultrastructure of SP-A, as judged by electron microscopy, is very similar to that of complement subcomponent C1q. The quantity of lung surfactant in alveolar spaces is controlled by regulation of secretion and removal (by phagocytosis) of surfactant. *In vitro* and *in vivo* studies indicate that SP-A is involved in regulating both uptake and secretion of surfactant. In the presence of SP-A, the uptake of radiolabeled surfactant-like lipids, by alveolar type-II cells and alveolar macrophages is increased by up to tenfold [6]. Dobbs et al. [7] showed that addition of SP-A into cultures of alveolar type-II cells containing ^{3}H-labeled choline reduced the secretion of ^{3}H-labeled phosphatidylcholine (a marker for surfactant secretion).

Adherence of macrophage to SP-A-coated surfaces has been shown to enhance Fc receptor- and complement receptor type 1-mediated phagocytosis of particles opsonized with antibody and complement [8]. Van-Iwaarden et al. [9] showed that the phagocytosis by rat alveolar macrophage of *Staphylococcus aureus*, pretreated with rat serum, was enhanced in the presence of SP-A. Kuroki et al. [10] and Wright et al. [11] reported characteristics of the binding of SP-A to a receptor on alveolar type-II cells. The binding of radioiodinated SP-A to alveolar type-II cells was not inhibited by mannose, indicating that the C-type lectin domain in the globular region of SP-A was not involved. Similarly Weber et al. [12] provided evidence for interaction of SP-A with dog alveolar macrophage and polymorphs via the collagenous domain. Interaction of SP-A with cells can, however, also be mediated by the Ca^{2+}-dependent lectin domains, as shown by Wintergerst et al. [13]. The structurally similar protein, C1q, is also involved in phagocytic uptake processes [8, 14, 15] and binds to a receptor on macrophages and other cell types via its collagenous region [16]. The similarities in ultrastructure and activities of SP-A and C1q prompted further examination of their interaction with receptor(s). Earlier experiments indicated that the receptor for C1q, C1qR, binds specifically not only to C1q, but also to mannan-binding protein (MBP), conglutinin and lung surfactant protein A (SP-A) [17]. In this article we present detailed studies of the interaction of purified human SP-A with purified C1qR and with the C1qR-expressing monocytic cell line, U937 cells.

[I 9997]

* This project was founded by the British Lung Foundation and the Colt Foundation.
◇ Recipient of a British Lung Foundation fellowship.

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2 Materials and methods

2.1 Commercial materials

Iodogen was obtained from Pierce and Warriner (Chester, GB). Tissue culture medium was purchased from Flow

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Laboratories (Irvine, Ayrshire, Scotland). Calcium ionophore A23187, monensin and emulphogene BC 720 were purchased from Sigma Chemical Co. (Poole, GB). Lenzol immersion oil was purchased from BDH Limited (Poole, GB). Sepharose 4B and PD-10 Sephadex G-25 (medium grade) gel filtration columns were purchased from Pharmacia (Milton Keynes, GB).

2.2 Purification of proteins

C1qR was purified from tonsil lymphocytes by the method of Malhotra and Sim [18]. C1q was isolated from human serum as described by Reid [19]. Human SP-A was purified from human bronchoalveolar lavage fluid (Haurum and Thiel, in preperation). Bronchoalveolar lavage fluid was diluted in an equal volume of 20 mM Tris/HCl (pH 7.4) containing 10 mM $CaCl_2$, 10 mM NaCl and 0.1% (v/v) emulphogene BC-720. The sample was loaded on to a Sepharose 4B column preequilibrated in 10 mM Tris/HCl buffer (pH 7.4) containing 5 mM $CaCl_2$, 10 mM NaCl and 0.1% emulphogene. The column was washed with equilibration buffer and the bound SP-A was eluted with 10 mM Tris/HCl buffer (pH 7.4) containing 10 mM EDTA, 10 mM NaCl and 0.1% emulphogene. On SDS-PAGE, under reducing conditions, SP-A migrated as a single component of 30–32 kDa. Assuming that each molecule of SP-A is composed of 18 polypeptide chains the relative molecular mass of SP-A in solution was taken to be 576 kDa.

2.3 Radioiodination of purified proteins

Radioiodination was carried out by the Iodogen method [20] with 1 mCi of $Na^{125}I$ for radioiodination of 0.5 ml of protein solution (10–20 μg/ml) in 10 mM sodium phosphate buffer, pH 7.4. Excess free ^{125}I was removed by desalting on a PD-10 Sephadex G-25 (medium grade) gel filtration column run in the same buffer.

2.4 Binding of ligands to U937 cells

U937 cells were grown in 50-ml culture flasks in standard conditions [21]. U937 (10^8 cells) were washed three times with phosphate-buffered saline, followed by three washes with 5 mM sodium barbitone buffer containing 5% (w/v) glucose, pH 7.4 (buffer A). The cells were resuspended in 10 ml of buffer A.

For binding assays different dilutions of radioiodinated SP-A (final concentration 14 nM; specific activity 3.5×10^6 cpm/μg of SP-A) were incubated with 100 μl of U937 cells (10^6 cells) for 30 min at 37°C. Unbound ligand was separated by spinning the cell suspension through 150 μl of lenzol in a 0.4-ml microcentrifuge tube for 15 s at $10000 \times g$ in a microfuge. Radioactivity bound to the cell pellet was measured.

For competition binding assays a constant concentration of radiolabeled SP-A (14 nM; specific activity 3.5×10^6 cpm/μg of SP-A) was preincubated with different dilutions of potential binding competitors including C1q (50 μl; maximum concentration 80 μg/ml), C1qR (50 μl; maximum concentration 40 μg/ml) or bovine serum albumin (50 μl; maximum concentration 50 μg/ml) for 30 min at 37°C in buffer A. The mixture was incubated with 100 μl of U937 cell suspension (10^6 cells) for 30 min at 37°C. Radioactivity bound to the cell pellet in the presence or absence of the competitor was measured.

2.5 Effect of ionic strength on binding of SP-A to U937 cells

A constant concentration of radiolabeled SP-A (1 nM; 100 μl; specific activity 4.3×10^7 cpm/μg of SP-A) was incubated with 100 μl of U937 cell suspension (10^6 cells) at 37°C for 60 min in 5 mM sodium barbitone buffer, pH 7.4, containing different concentrations of sodium chloride (isotonicity of the buffer was maintained by adding the appropriate quantity of glucose). Radioactivity bound to the cell pellet was measured as above.

2.6 Time course of binding

A constant concentration of radiolabeled SP-A (1 nM; 100 μl; specific activity 4.3×10^7 cpm/μg) was incubated with 100 μl of U937 cell suspension (10^6 cells) in the presence or absence of sodium azide (5 mM) in buffer A at 37°C or 4°C for different time intervals. Radioactivity bound to the pellet was measured as above. In further experiments different concentrations of radiolabeled SP-A (maximum concentration 1 nM; 100 μl; specific activity 4.3×10^7 cpm/μg) were incubated with 100 μl of U937 cell suspension (2.5×10^5 cells) at 37°C for 60 min or 120 min. Radioactivity bound to the pellet was measured as above.

2.7 Effect of calcium ionophore A 23187 and monensin

U937 cell suspension (100 μl; 10^6 cells) in buffer A was preincubated with different dilutions of calcium ionophore (maximum concentration 11 μM) or monensin (maximum concentration 0.75 mM) for 10 min at 37°C. The mixture was then exposed to radiolabeled SP-A (1 nM; 100 μl; specific activity 4.3×10^7 cpm/μg) for different time intervals. The radioactivity bound to the cell pellet was measured as above. In a further experiment U937 cells (10^6 cells) were preincubated with a constant concentration of calcium ionophore (11 μM) or monensin (0.1 mM) in buffer A or in buffer A alone for 10 min at 37°C. Radiolabeled SP-A (1 nM; 100 μl; specific activity 4.3×10^7 cpm/μg of SP-A) was added to the mixture and the time course of binding was followed. Radioactivity bound to the pellet was measured as above.

2.8 Background subtraction for binding experiments

Freshly radioiodinated SP-A gave low background (nonspecific) binding, as seen in Fig. 1. On storage at 4°C for up to 3 weeks, the radiolabeled exhibited an increase in nonspecific binding. When freshly iodinated material was used for binding studies, no background subtraction was applied to the binding curves (*e.g.* Fig. 1). When, however, older material was used, the background was caculated by determining binding of ^{125}I-labeled-SP-A in the presence of

a 100-fold molar excess of unlabeled C1q (Figs. 2, 3 and 5). This was not ideal, as it would have been preferable to use excess unlabeled SP-A; however, insufficient SP-A was available for this purpose. C1qR is a widely distributed receptor, and a suitable receptor-negative cell line was not available for background subtraction.

3 Results

3.1 Binding of SP-A to U937 cells

Binding of SP-A to U937 cells was generally examined at low ionic strength, for comparison with the C1q-C1qR system, in which binding of monomeric ligand is more readily measurable at low ionic strength [17]. Further, binding was generally studied in the absence of calcium ions, to eliminate possible binding via the lectin domain. U937 cells (10^6 cells) were incubated with different concentrations of ^{125}I-labeled-SP-A for 30 min at 37°C in 5 mM sodium barbitone buffer containing 5% glucose. Concentration-dependent and saturable binding of radiolabeled SP-A to U937 cells was observed (Fig. 1a). Scatchard analysis of the binding data, assuming the molecular weight of SP-A is 576000, indicated a dissociation constant of $7.4 \pm 0.1 \times 10^{-10}$ M (Fig. 1b). We reported earlier that the binding of radiolabeled C1q to U937 cells is inhibited in a concentration-dependent manner by SP-A, MBP and conglutinin [17]. To show that the receptor involved in the binding of SP-A to U937 cells is the same as the one characterized [18] for C1q, the binding of radiolabeled SP-A was examined in the presence of different concentrations of C1q or of purified C1qR. Concentration-dependent inhibition of binding of radiolabeled SP-A to U937 cells was observed both in the presence of C1q and purified C1qR (Fig. 1c), whereas bovine serum albumin did not affect the binding (Fig. 1c). These results indicate that the binding site for SP-A on U937 cells is same as that for C1q and that the molecule isolated by us as C1qR is the most probable candidate for this binding.

3.2 Effect of ionic strength on binding of radiolabeled SP-A to U937 cells

Binding of monomeric C1q to U937 cells is ionic strength dependent and does not require Ca^{2+} ions [22]. In Fig. 2 is shown the effect of ionic strength on binding of SP-A to U937 cells. The binding of SP-A to U937 cells was maximum at low ionic strength, and is very similar to the binding behavior of C1q to C1qR [22].

3.3 Time course of binding of radiolabeled SP-A to U937 cells

Initial binding of ligand to U937 cells was rapid (Fig. 3a) and occurred within 5 min. A gradual increase in binding was observed between 5 min and 60 min, but between 60–80 min a further marked increase in binding of radiolabeled SP-A to U937 cells was observed (Fig. 3a). The time course experiment was repeated in the presence or absence

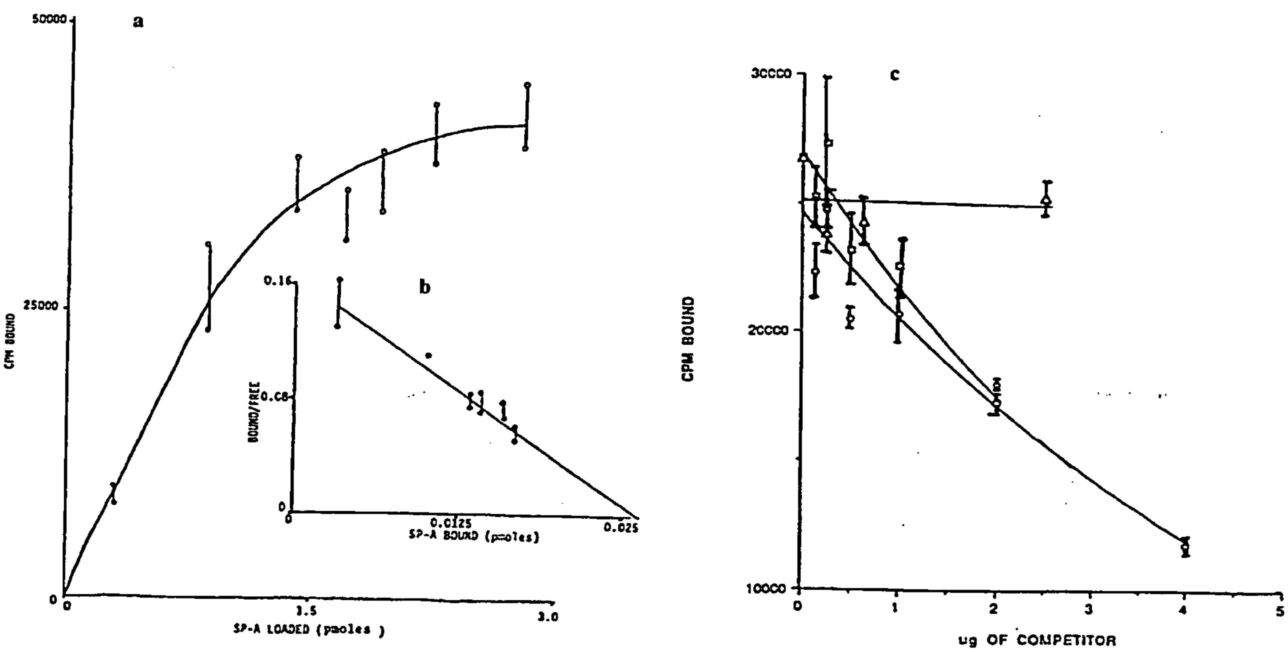


Figure 1. Binding of SP-A to U937 cells and competition with C1q and C1qR. (a) Different dilutions of radioiodinated SP-A were incubated with of U937 cells (10^6 cells) for 30 min at 37°C in buffer A. Unbound ligand was separated by spinning the cell suspension through lenzol and the bound radioactivity was measured. Details are given in Sect. 2.4. The direct binding curve is shown. Results of a single experiment with the average and the range of triplicate experimental points is shown by error bars. (b) Scatchard plot obtained from the binding data. (c) Radiolabeled SP-A was preincubated with different dilutions of potential competitors C1q (O), C1qR (□) or bovine serum albumin (Δ) for 30 min at 37°C in buffer A as described in Sect. 2.4. The mixture was incubated with U937 cell suspension (10^6 cells) for 30 min at 37°C. Radioactivity bound to the cell pellet in the presence of the competitor was measured. Results of single experiments with the average and range of triplicate experimental points is shown by error bars.

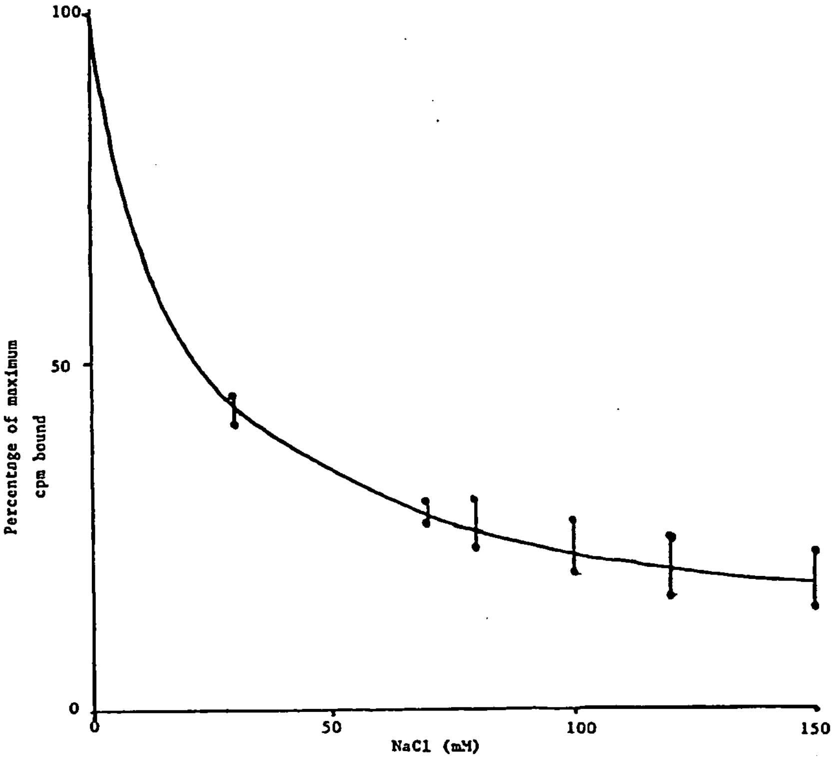
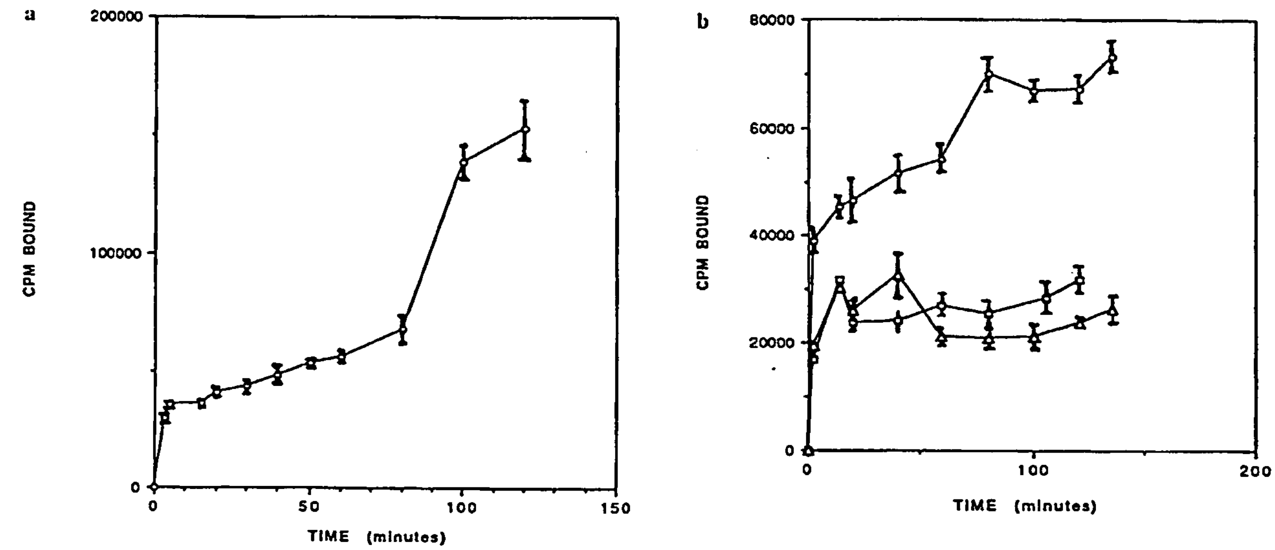


Figure 2. Effect of ionic strength on SP-A binding to U937 cells. A constant concentration of radiolabeled SP-A was incubated with 100 µl of U937 cell suspension (10^6 cells) at 37 °C for 60 min in different concentrations of sodium chloride as described in Sect. 2.5. Background values were subtracted before calculating the percentage of maximum cpm bound. Results of a single experiment with the average and range of triplicate experimental points is shown by error bars.

of sodium azide (5 mM) or excess cold C1q (final concentration 100 µg/ml) or at 4 °C. The sudden increase in binding of ligand between 60–80 min was not observed in the presence of sodium azide or at 4 °C (Fig. 3 b), and the binding of SP-A to U937 cells was inhibited throughout the time course by the presence of excess C1q (Fig. 3 c). The increase in binding of SP-A to U937 cells could arise in several ways. There may be internalization of SP-A followed by recycling of receptor to allow further binding, or the increase may be due to the changes in binding affinity, or to increase in expression of C1qR on the cell surface. To investigate these possibilities, U937 cells were incubated with different concentrations of radiolabeled SP-A at 37 °C for 60 min or for 120 min. The Scatchard plots (Fig. 4 a) generated from the binding data show a shift in the plot, but no change in the slope of the plot for the two stated time invervals, indicating that the increase in binding is due to apparent increase in receptor number (from 6000 receptor sites/cell to 8000 receptor sites/cell) with no change in binding affinity (dissociation constant of $7.4 \pm 0.1 \times 10^{-10}$ M). The results shown in Fig. 4 a do not distinguish between true increase in surface receptor number, and apparent increase due to internalization of ligand (*i.e.* increase in total ligand associated with the cell). An attempt to dissociate surface bound SP-A using excess C1q (Fig. 4 b) indicated that after 120 min of incubation with U937 cells, SP-A is less extensively dissociated than after 60 min of incubation. This, in turn, suggests that some SP-A may be internalized and unavailable for competitive dissociation by C1q.

3.4 Effect of calcium ionophore A23187 and monensin on binding of radiolabeled SP-A to U937 cells

Calcium ionophore has been reported to induce exocytosis and expression of intracellular pools of receptors by transporting Ca^{2+} ions across the plasma membrane [23]. To show whether the increase in ligand binding observed in Figs. 3 and 4 arises from the expression of an intracellular pool of the receptor as well as to ligand internalization, the U937 cells were incubated with different dilutions of calcium ionophore in the presence of 10 mM Ca^{2+} for 30 min at 37 °C, followed by addition of radiolabeled SP-A. The binding of radiolabeled SP-A was assessed after 5 min, 40 min and 120 min of incubation at 37 °C. In Fig. 5 a is shown the effect of preincubation of cells with calcium ionophore on the binding of ligand to U937 cells. An increase, dependent on ionophore concentration, in binding of SP-A to U937 cells at the 5 min and 40 min time points was observed in the presence of calcium ionophore, whereas at 120 min the binding was relatively constant for the different concentrations of ionophore. To establish further the effect of calcium ionophore on binding of SP-A to U937 cells, a time course for binding of SP-A to U937 cells was done with the cells being preincubated for 30 min at 37 °C with or without calcium ionophore (11 µM). The results in Fig. 5 b show that calcium ionophore in the presence of 10 mM Ca^{2+} increased the level of binding of SP-A to the cells at early time points. The results shown in Fig. 5 a, b strongly suggest that preincubation of cells with calcium ionophore induces rapid increase in surface expres-



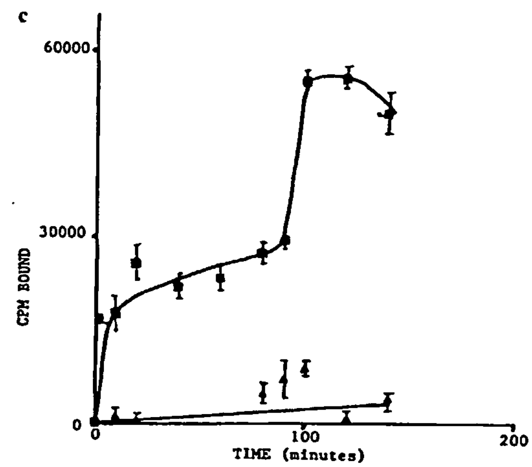


Figure 3. Time course of binding of SP-A to U937 cells. (a) A constant concentration of radiolabeled SP-A was incubated with 100 µl of U937 cell suspension (10^6 cells) for different time intervals in buffer A as described in Sect. 2.6. At a given time the cells were removed and spun through oil to remove the unbound ligand. The radioactivity bound to the pellet was measured. Results of typical experiment with the average and range of triplicate experimental points is shown by error bars. The time course experiment was repeated at 4 °C (△) (b) or at 37 °C in the presence (□) or absence (○) of sodium azide (5 mM) (b) or in the presence (▲) or absence (■) of C1q (final concentration 100 µg/ml) (c). Results of single experiments with the average and range of triplicate experimental points is shown by error bars.

sion of SP-A binding sites. The 10-min preincubation with calcium ionophore therefore apparently causes an increase in surface expression of C1qR similar to that seen (Fig. 3) after prolonged incubation of cells with SP-A. This, in turn, may indicate that SP-A itself induces increase in receptor number, possibly by increasing intracellular Ca^{2+} ions.

To examine further the recycling of C1qR, the cells were preincubated with different concentrations of monensin, and then incubated with radiolabeled SP-A for different time intervals. Monensin has been shown to increase endosome pH and cause a major disruption of the transport of membrane vesicles from the Golgi complex to the plasma membrane [24]. Increasing concentration of monensin effectively abolished the rise in ligand binding normally seen after 60–80 min (Fig. 5 c), suggesting that transport or recycling of internal C1qR was disrupted. This is shown with a single monensin concentration in Fig. 5 d. Preincubation of cells with monensin reduced the expression of C1qR and inhibited the sudden increase in receptor number observed in the normal time course.

4 Discussion

Lung SP-A is the major protein component of lung surfactant and has been reported to regulate the secretion of surfactant. As in C1q, the polypeptide chains of SP-A consist of a short N-terminal segment, followed by a region of collagen-like sequence (characterized by the repeating triplet sequence Gly-X-Y, where Y is often a hydroxylated amino acid). The C-terminal half of the sequence is non-collagenous, and contains, in SP-A, a structure known as a C-type lectin domain, indicating that the protein exhibits calcium ion-dependent binding to carbohydrates [25]. Subunits of the protein are built up by association of three polypeptide chains, the collagenous regions of which intertwine to form a collagen triple helix. The non collagenous C-terminal halves form a globular "head". The collagen triple helix is bent in each subunit, due to an interruption in the repeating Gly-X-Y sequence in each polypeptide chain. Six such subunits associate to form the characteristic "bunch of tulips" structure seen in electron microscopy of C1q or SP-A. In this structure, the globular

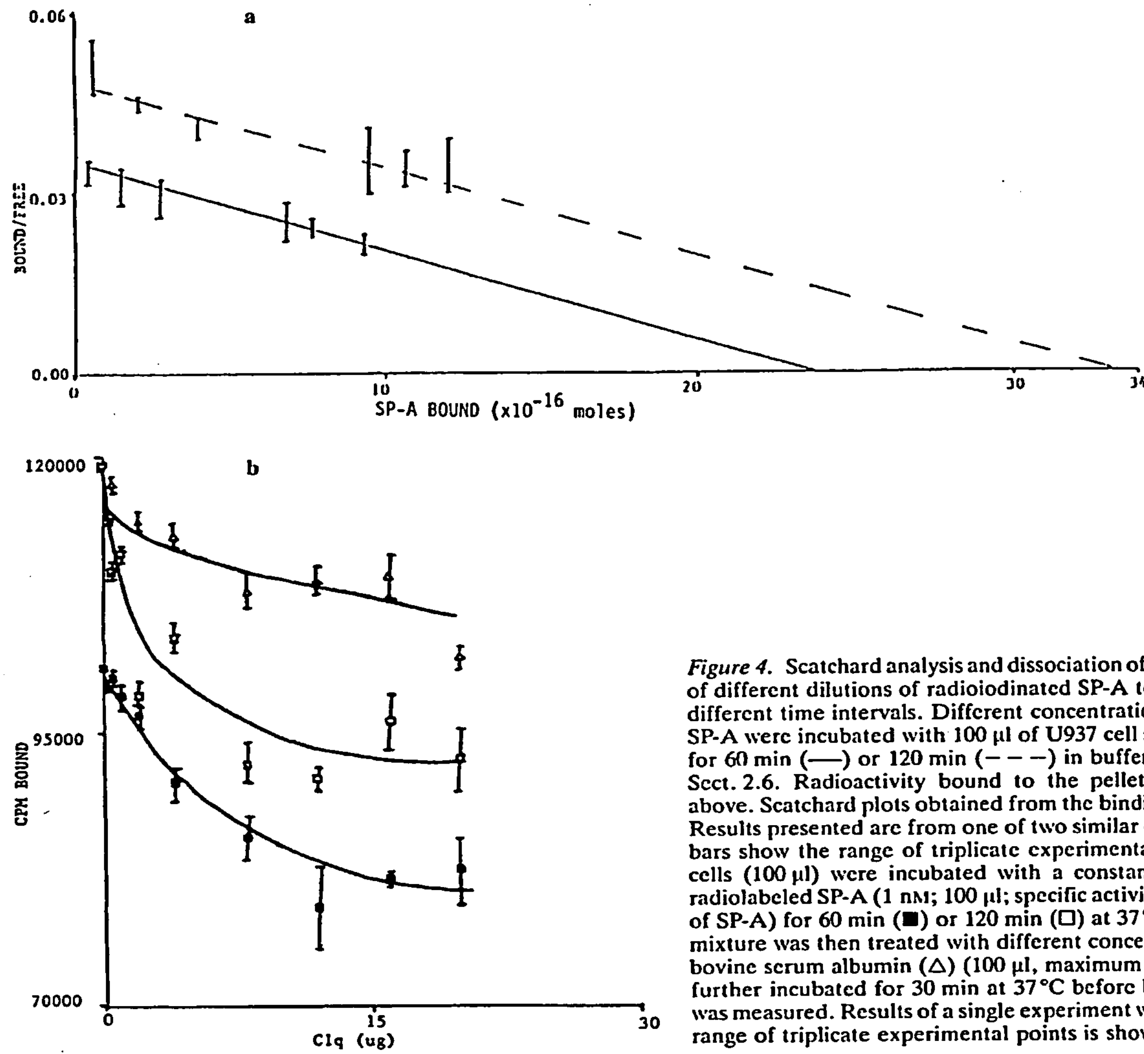
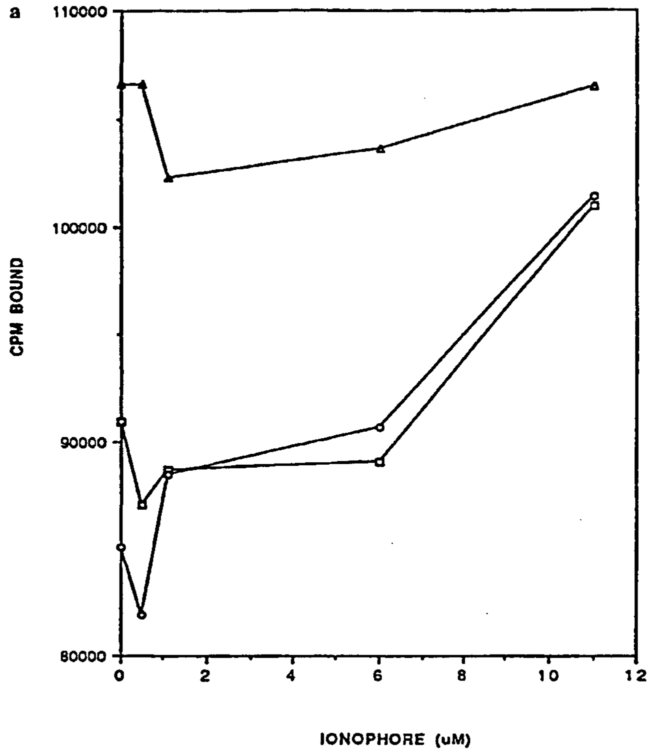
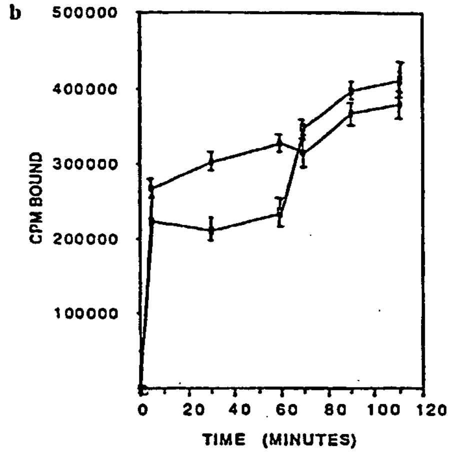


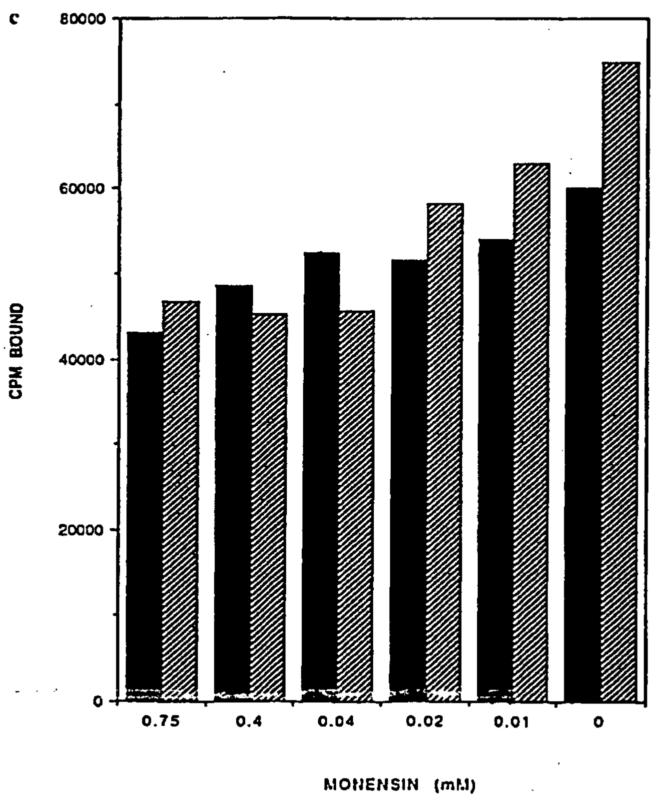
Figure 4. Scatchard analysis and dissociation of ligand. (a) Binding of different dilutions of radioiodinated SP-A to U937 cells at two different time intervals. Different concentrations of radiolabeled SP-A were incubated with 100 µl of U937 cell suspension at 37 °C for 60 min (——) or 120 min (– – –) in buffer A as described in Sect. 2.6. Radioactivity bound to the pellet was measured as above. Scatchard plots obtained from the binding data are shown. Results presented are from one of two similar experiments. Error bars show the range of triplicate experimental points. (b) U937 cells (100 µl) were incubated with a constant concentration of radiolabeled SP-A (1 nM; 100 µl; specific activity 4.3×10^7 cpm/µg of SP-A) for 60 min (■) or 120 min (□) at 37 °C in buffer A. The mixture was then treated with different concentrations of C1q or bovine serum albumin (△) (100 µl, maximum amount 20 µg) and further incubated for 30 min at 37 °C before bound radioactivity was measured. Results of a single experiment with the average and range of triplicate experimental points is shown by error bars.

heads form the "flowers", and the collagen helices form the stalks [25] Dobbs et al. [7] showed that SP-A inhibited the basal level of secretion of surfactant and reduced to basal levels secretion stimulated by terbutaline, phorbol 12-myristate 13-acetate and the ionopore A 23187. This effect was shown to be blocked when SP-A was preheated to 100 °C for 10 min or in the presence of antiserum specific to SP-A. Tenner et al. [8] showed that monocytes or macrophages attached to SP-A-coated plates were much more effective in uptake of IgG- or IgM- or complement-coated sheep erythrocytes, than were the free monocytes or macrophages. The enhancement was dependent on the concentration of SP-A. These observations were further supported by Van Iwaarden et al. [9], who showed that phagocytosis of *Staphylococcus aureus* treated with rat serum by rat alveolar macrophages was enhanced in the presence of SP-A. These observations indicate receptor-mediated interaction of SP-A with alveolar type-II cells, macrophages and monocytes. The binding characteristics of SP-A with an SP-A receptor on human cell lines have not been extensively investigated, but a number of workers have characterized the binding of SP-A to rat alveolar type-II cells and rat alveolar macrophages. Rice et al. [26] showed that an SP-A peptide corresponding to the carbohydrate-binding lectin domain did not inhibit ATP-stimulated secretion of surfactant by rat alveolar type-II cells. Kuroki et al. [10] showed that mannose or α-methyl mannoside or concanavalin A did not effect the binding of radioiodinated rat SP-A to alveolar type-II cells, nor did these substances affect the SP-A-induced inhibition of surfactant secretion. They also showed that chemical modification of basic amino acids in SP-A resulted in inhibition of binding of radioiodinated SP-A to alveolar type-II cells and in inhibition of biological activity of SP-A. Wright et al. [11] also reported that binding of radiolabeled SP-A to rat alveolar type-II cells was not inhibited by potentially competing sugars and lectins, indicating that the binding of SP-A to rat alveolar cells is independent of the lectin domain. Interaction of dog SP-A with dog alveolar macrophages and polymorphonuclear cells is destroyed by collagenase treatment [12].

In contrast to these findings Wintergerst et al. [13] reported that binding of SP-A, preabsorbed onto colloidal gold







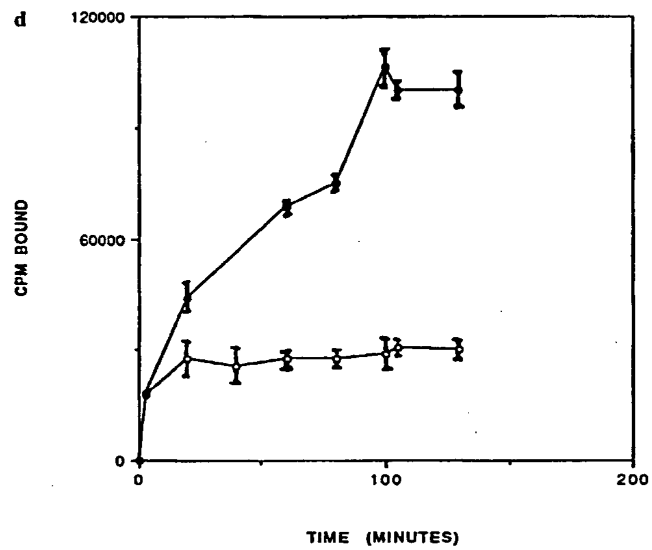


Figure 5. Effects of A23187 and monensin on binding. (a) U937 cells (10^6 cells) were preincubated with different dilutions of calcium ionophore for 10 min at 37 °C in buffer A. The mixture was then interacted with radiolabeled SP-A for 5 (□); 40 (○); or 120 min (△). Details are given in Sect. 2.7. Results of a single experiment with the average of triplicate experimental points is shown. (b) Interaction of radiolabeled SP-A (as in Fig. 5a) with U937 cells at 37 °C for different time intervals in the presence (●) or absence (□) of calcium ionophore (11 μM) in buffer A as described in Sect. 2.7. Results of a single experiment with the average and range of triplicate experimental points is shown. (c) U937 cells (10^6 cells) were preincubated with different dilutions of monensin (maximum concentration 0.75 mM) for 10 min at 37 °C in buffer A. The mixture was than interacted with radiolabeled SP-A (as in Fig. 5a) for 40 (■) or 100 min (▨). The radioactivity bound to the cell pellet was measured. (d) Interaction of radiolabeled SP-A with U937 cells (as in Fig. 5a) at 37 °C for different time intervals in the presence (○) or absence (●) of monensin. Details are given in the Sect. 2.7. Results of a single experiment with the average and range of triplicate experimental points is shown by error bars.

```
Human C1q(A chain)   GRPGLKGEQGEPGAPGIRT------------------------GIQGLKGDQG
Human C1q(B chain)   GQPGTPGIKGEKGLPGLA-------------------------GDHGEFGEKG
Human C1q(C chain)   GKDGYDGLPGPKGEPGIPAIP----------------------GIRGPKGQKG
Human MBP            GKDGRDGTKGIKGEPGQ--------------------------GLRGLQGPPG
Human SP-A           GRDGRDGLKGDPGFPGPMGPPGEMPCPP-----GNDGLPGAPG
```

Figure 6. Amino acid sequences of human C1q (A chain) [33], human C1q (B chain) [33], human C1q (C chain) [33], human MBP [34] and human SP-A [35] from a region around the middle of the collagen domain. The bend region of the collagen domain is indicated by (-----). The homology (identities and conservative changes) between the chains (ignoring repetitive glycines) is shown by boxes.

particles, to macrophages derived from blood monocytes was inhibited by α-D-mannosyl-BSA. These results suggest that the carbohydrate binding activity of SP-A is responsible for binding of SP-A to cells. The results reported by different workers indicate that there are two different types of binding sites available on the rat alveolar type-II cells and rat macrophages for SP-A binding. These two types of sites interact with either the lectin-like domain in the globular head of SP-A or an undefined region in the collagen-like domain. In this article we have presented evidence for an SP-A receptor on the human monocytic cell line U937. Data presented indicate that SP-A shows saturable and specific binding to U937 cells. The binding of radioiodinated SP-A is inhibited by C1q and soluble C1qR and is ionic strength dependent. Data presented here indicate that C1qR is a major binding site for SP-A on U937 cells. Similarity in binding characteristics and in ultrastructure between C1q and SP-A suggest that, like C1q, SP-A might play a major role in host defense mechanisms. C1q, other than acting as an activator of the classical pathway of the complement system, has been reported to mediate phagocytosis via C1qR, modulation of cytokine and immunoglobulin secretion and polymorphonuclear cell-endothelial cell interaction [27, 28]. The interaction of C1q with C1qR occurs via the collagen-like domain of C1q [16, 29]. The C-terminal globular head of SP-A contains a C-type lectin domain and this domain has been reported to bind phospholipids [30] and carbohydrates [31]. The C-terminal globular domain of C1q binds IgG and other species, and is not homologous to the head of SP-A. There is only limited sequence similarity between the collagenous segments of C1q and SP-A: the amino acid sequence close to the bend region in the collagen-like domain appears to contain a high proportion of charged residues (Fig. 6). As the binding of SP-A or C1q to C1qR is highly salt dependent and chemical modification of basic residues in SP-A has been shown to inhibit the binding of SP-A to rat alveolar type-II cells [10], we suggest that the region close to the collagen triple helix bend region might be the binding site for SP-A or C1q with C1qR. Other known ligands for C1qR are MBP and conglutinin. Both of these, as with C1q and SP-A, have similar overall structure, being made up of many small polypeptides, each with an N-terminal region of collagen-like sequence, and a C-terminal non-collagenous segment. These polypeptides associate in groups of three to form a collagen triple helix and a three-loped globular head. These structures then associate to form covalent or non-covalent trimers-hexamers. MBP and conglutinin, like SP-A have C-type lectin domains in the C-terminal half of each polypeptide. MBP shares the feature discussed in Fig. 6. Other partially characterized proteins including SP-D and RaRf have similar overall structure. All these proteins can be grouped into a family which contain in their primary sequence a collagen-like sequence associated with non-collagenous sequence. As the proteins associated with this group have a collagen-like domain and/or associate with a complement receptor and have been reported to bind carbohydrates (lectin activity) [25], we propose the name COLLECTIN for this family of proteins.

The time course for the binding of radiolabeled SP-A to U937 cells indicates that ligand binding to cells induces surface expression of an intracellular pool of C1qR. This effect was also induced by calcium ionophore in the absence of ligand. Calcium ionophore has been reported to transport Ca^{2+} from the extracellular fluid into the cell, thus increasing the intracellular Ca^{2+} concentration, which in turn induces the exocytosis or expression of intracellular pools of cell surface molecules [23]. These results suggest that increased expression of C1qR in the presence of SP-A is induced by increase in intracellular Ca^{2+} ions and it has been reported earlier that interaction of C1q with C1qR induces increase in cytosolic Ca^{2+} in mouse fibroblasts [32]. Further evidence for recycling of C1qR was provided by the effect of monensin on the expression of C1qR on U937 cells. Monensin completely inhibited the increase in binding of radiolabeled SP-A with U937 cells observed in the time course and reduced the receptor number. Monensin, a carboxylic acid ionophore, has been shown to increase endosome pH and cause a major disruption of the transport of membrane vesicles from the Golgi complex to the plasma membrane [24]. Binding of radiolabeled SP-A to U937 cells in the time course was also inhibited, when the time course was done in the presence of sodium azide or when the cells were incubated at 4 °C. All these strongly indicate recycling of C1qR, which might involve transport of C1qR from Golgi to plasma membrane and this transport is triggered by increase in cytosolic Ca^{2+} concentration.

We thank Miss B. Moffatt and Mrs. J. U. Newell for technical assistance. We also thank Dr. K. B. M. Reid for useful discussion and advice.

Received October 8, 1991; in revised form December 20, 1991.

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REVIEW

Collectins—soluble proteins containing collagenous regions and lectin domains—and their roles in innate immunity

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(RECEIVED March 17, 1994; ACCEPTED May 5, 1994)

Abstract

The collectins are a group of mammalian lectins containing collagen-like regions. They include mannan binding protein, bovine conglutinin, lung surfactant protein A, lung surfactant protein D, and a newly discovered bovine protein named collectin-43. These proteins share a very similar modular domain composition and overall 3-dimensional structure. They also appear to play similar biological roles in the preimmune defense against microorganisms in both serum and lung surfactant. The close evolutionary relationship between the collectins is further emphasized by a common pattern of exons in their genomic structures and the presence of a gene cluster on chromosome 10 in humans that contains the genes known for the human collectins. Studies on the structure/function relationships within the collectins could provide insight into the properties of a growing number of proteins also containing collagenous regions such as C1q, the hibernation protein, the α- and β-ficolins, as well as the membrane acetylcholinesterase and the macrophage scavenger receptor.

Keywords: collagen-like; collectins; innate immunity; lectin; mannan binding protein; lung surfactant protein

A growing number of mammalian plasma and cell-surface proteins have been found to contain collagen-like regions attached to noncollagenous domains; however, they are not included in the definition of "collagens," which exclusively refers to proteins of the extracellular matrix. The proteins with collagenous domains found in other locations include not only the "collectins," but also 6 other proteins, namely serum complement protein C1q (Brodsky-Doyle et al., 1976; Sellar et al., 1991), α- and β-ficolin (Ichijo et al., 1993), the "hibernation protein" from squirrel (Takamatsu et al., 1993), the macrophage scavenger receptor (Kodama et al., 1990), and the membrane acetylcholinesterase (MacPhee-Quiley et al., 1986; Krejci et al., 1991). Among these collagenous molecules are the collectins, which form a family of oligomeric proteins, which contain a C-type lectin domain at the C-terminal end of each of their polypeptide chains. The collectins include serum mannan binding protein (MBP), bovine conglutinin and collectin-43 (CL-43), and the lung surfactant proteins SP-A and SP-D (Holmskov et al., 1994). The structure/function relationships within the collectins are at present under intensive study and it is becoming clear that the C-type lectin domains, by binding to carbohydrate ligands on the cell-surface of pathogens, fulfill a recognition function that can elicit effector functions via the collagen-like region, such as complement activation (in the case of MBP) or binding to cell-surface receptors to trigger phagocytosis or oxidative killing. These relationships resemble closely the well-established correlation between the structure of C1q and its functions, such as binding to immune complexes, activating complement and interacting with cell-surface receptors, with the collagen-like regions and the globular domains playing very distinct roles (Reid, 1983; Tenner, 1993). Because some of the collectins display similar functions to C1q, but do so without the involvement of antibody, it is probable that they play an important role in innate immunity.

The overall domain organization seen in the collectins is illustrated in Figure 1A. Four distinct regions can be identified within these proteins, three of which are structurally well defined. An independently folding C-type lectin domain is located at the C-terminal end of each polypeptide chain (Drickamer, 1988). The lectin domain is preceded by a stretch of about 40 amino acids, which have the potential to form a 3-stranded

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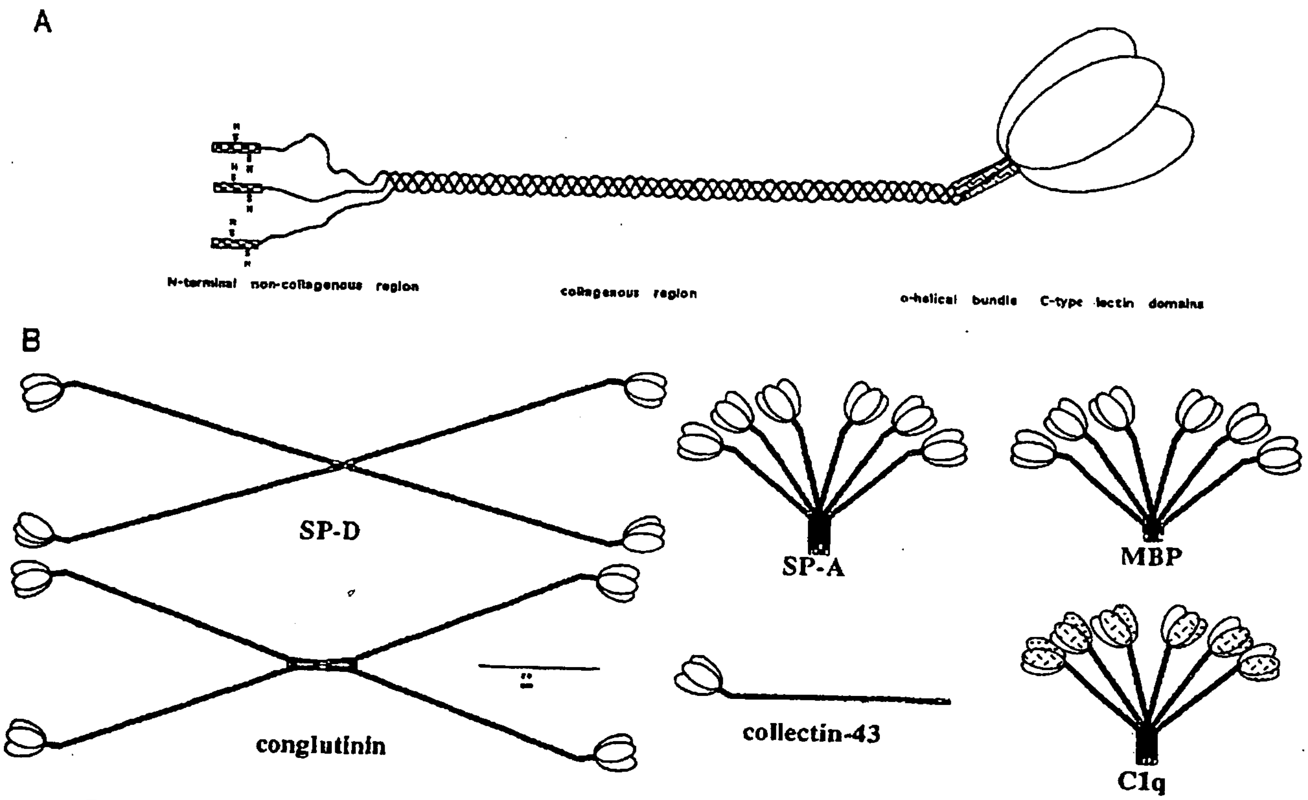


Fig. 1. Domain organization and assembly of the collectin molecules and C1q. A: Three polypeptide chains, each having a C-terminal C-type lectin domain, are considered to associate through strong hydrophobic interactions via the α-helical bundle forming the "neck" region. The collagen triple-helix forms in a zipper-like fashion from the C-terminal end to the N-terminal end, where a short noncollagenous region is believed to mediate oligomerization, involving disulfide bridges, to the higher oligomers shown in B. B: Schematic drawings of C1q and the molecules belonging to the collectin family of proteins (drawn to scale). The overall dimensions were determined by electron microscopy. However, very few side views are available of SP-A to allow the estimation of the length of the collagenous parts, which appear aligned in parallel at the foot of the molecule and thus hold the 6 triple-helical rods together to give a hexameric structure. No oligomers have as yet been found in the case of collectin-43. The globular domains of the 3 chains of C1q are not identical.

α-helical coiled-coil when associated with the equivalent regions in 2 other chains (Hoppe et al., 1994). The third domain is the collagenous region, which is always found directly N-terminal to the α-helical bundle. The repeating Gly-Xaa-Yaa triplet regions from 3 chains of any collectin form a collagen triple-helix of varying length. In SP-A and MBP, the sequences include an interruption, at triplets 13 and 8, respectively, and therefore the collagen helices are considered to bend at these points (Voss et al., 1988; Fig. 1B). The N-terminal portion in each molecule is short, containing at least 1 cysteine residue per chain, and these cysteines are thought to play an important role in the assembly of the trimeric, collagen-containing rods into higher oligomers (Fig. 1B). However, little is known about the secondary structure of this region.

The collectins are considered to act as a "bridge" between very large particles, which have on their surface certain exposed carbohydrates (Lu et al., 1990) (such as those found on invading microorganisms or, indeed, allergens derived from house dust mites or pollen grains), and the host's immune system. Their functions can be divided into 2 categories, namely those that rely on the carbohydrate recognition specificities of the lectin domains involved and those of the subsequent interactions with the host's effector systems or cells (Malhotra et al., 1990).

The collectins have been characterized at the cDNA and the genomic levels and the sequences, and intron/exon positions, are consistent with the domain organization outlined in Figure 1. Bacterial expression of distinct regions of the collectins has been crucial to the structure determination of both the C-type lectin domain (of MBP) and the 3-stranded α-helical bundle (of human SP-D). Analysis of the gene sequence of MBP suggests that the opsonic defects seen in infants suffering from recurrent infections correlate with point mutations in the MBP genes, a finding that may have important implications for the analysis of inflammatory disorders of the lung, possibly involving the collectins SP-D and SP-A and their genes.

Structure of the collectins

The lectin domain

Lectins are defined as proteins other than antibodies or enzymes that bind carbohydrates (Barondes, 1988). The specificity of a

given lectin is usually determined by the method of binding-site saturation in which different sugars are tested for their ability to inhibit lectin binding to immobilized carbohydrates and is defined in terms of the most potent carbohydrate inhibitor. Animal lectins are not a homogeneous class of proteins, but can be divided into 2 major groups: the C-type and the S-type lectins (Drickamer, 1988). C-type lectins are extracellular proteins that bind to a variety of carbohydrates in a strictly Ca^{2+}-dependent manner, whereas S-type lectins have no requirement for divalent cations; they occur both intra- and extracellularly and bind predominantly β-galactosides (Barondes, 1984; Liao et al., 1994). The main exceptions, in terms of carbohydrate binding proteins, which do not fit into either of these categories of lectins, are the extracellular proteins laminin and fibronectin (Yamada, 1983); the mannose-6-phosphate receptor (Kornfeld, 1986), responsible for targeting proteins to the lysosomal compartments; and the viral hemagglutinins (Wiley & Skehel, 1987). The pentraxin C-reactive protein (Kolb-Bachofen, 1991; Kottgen et al., 1992) and serum amyloid component P (SAP) (Kubak et al., 1988) also show lectin activities. The crystal structure of SAP has recently been determined and a high degree of similarity was seen in comparison to the 3-dimensional structure of legume lectins, such as concanavalin A (Emsley et al., 1994).

Collectins contain lectin domains of the calcium-dependent type that are between 114 and 118 amino acids long with 14 invariant and 18 highly conserved residues, including 4 cysteine residues giving rise to the disulfide pattern 1-4 and 2-3 (Drickamer, 1988). C-type lectin domains occur in a wide variety of modular proteins (Table 1). The 3-dimensional structure of the recombinant C-type lectin domain of rat MBP has been determined by X-ray crystallography (Weis et al., 1991b) and was found to have the following features: (1) the N- and C-terminal ends of the lectin domain are located very close to one another, thus making it an ideal domain to link to the N- or C-terminal end of a polypeptide chain; and (2) the structure appears divided by 2 transverse β-strands that separate a compact scaffold of 2 helices and 2 β-sheets, with the N- and C-termini, from 1 very extensive loop, carrying the binding sites for 2 calcium ions as well as for the carbohydrate ligand. Thus, half of the domain, consisting of nonregular secondary structure, is responsible for carbohydrate binding and is attached to a very tight core structure. One of the calcium ions bound by the lectin domain was shown in the crystal structure to be complexed by both conserved residues within the lectin domain and the 3 and 4 hydroxyl groups of the terminal α-D-mannose residue bound by the domain (Weis et al., 1992). Modeling different carbohydrate structures onto the binding site of the MBP lectin domain showed the importance of other residues in the domain, which are in close proximity to the binding site and sterically restrict the access of differently shaped carbohydrates. However, the binding site is not buried within the protein structure but appears to be located at the surface of the 2.5 × 2.5 × 4.0-nm lectin domain, allowing for binding to accessible sugars contained within complex oligosaccharide chains as well as recognition of terminal nonreducing sugars. However, in contrast to the interactions seen in S-type and legume lectin-type protein–carbohydrate binding, the crystal structure of the C-type lectin domain of MBP provided little evidence for van der Waals or hydrophobic interactions with the complexed carbohydrate.

The molecular architecture of C-type lectin domains permits site-specific alterations to be made in the carbohydrate-binding region, within the flexible loop, which do not appear to affect the integrity of the structural scaffold holding the loop in place (Quessenberry & Drickamer, 1991). Such alterations can transfer a galactose-binding activity into the mannose-binding protein C-type lectin domain (Drickamer, 1992). Two of the 5 residues dues responsible for complexing the calcium ion involved in carbohydrate binding, namely E185 and N187 within the sequence EPN-E-WND (where E185, N187, E193, N205, and D206 are considered to be of importance in binding Ca^{2+}), were shown to be predominantly involved in determining the specificity for hydroxyl groups in positions equivalent to the 3- and 4-OH groups of D-mannose ($^4C_1$ form) in monosaccharide recognition (Drickamer, 1992). Change of these 2 residues to Q185 and D187 resulted in a large increase in the affinity for D-galactose as opposed to D-mannose seen for the E/N wild type, reflecting the steric requirements of bi-equatorial vs. equatorial-axial positioned hydroxyl groups at positions 3 and 4 of the ligand. The correlation between specificity and residue pair was found in sequence comparisons to be in excellent agreement with other C-type lectin domains of known sequence and monosaccharide specificity (Table 1). Point mutations as well as peptide/mAb inhibition studies (Geng et al., 1991, 1992) were also used to localize the binding site(s) within the E- and P-selectin molecules (Erbe et al., 1992, 1993). However, the possibility of more than 1 binding site present within these adhesion molecules, or additional protein–protein interactions involved in selectin–glycoprotein recognition, cannot be ruled out, and should also be considered in the definition of ligands for the collectins.

Using the criteria discussed above in sequence alignments, the collectins MBP, conglutinin, CL-43, and SP-D were all found to belong to the mannose-type carbohydrate binding family, whereas SP-A assumes a hybrid position between the rest of the collectins and the domains of other lectins specific for galactose-type sugars, e.g., the cartilage proteoglycans, which is in good agreement with the observation that both types of sugars, galactose and mannose, can inhibit SP-A binding equally well (Haagsman et al., 1987). The macrophage galactose-receptor (Sato et al., 1992) and the Kupffer cell fucose receptor (Hoyle & Hill, 1988) also exhibit galactose-type selectivity, however they contain within their binding regions a 4-amino acid-long insertion between the 2 residues found to direct mannose-type or galactose-type hydroxyl-group specificity and residue E193. This may influence the conformation of the lectin domain at the binding site and thus define the steric requirements determining the binding activity for the natural polysaccharide ligands of these proteins. A similar observation can be made in case of the selectins (Lasky et al., 1989), which have a highly charged and conserved 5-residue-long insertion between E193 and N205/D206. Therefore, although the requirement for hydroxyl-groups of the mannose- or galactose-type within the binding-site on the carbohydrate molecules is determined by the E-N/Q-D pair of residues, other differences seen in the sequences of the lectin domains, and of the extensive loop carrying the binding sites in particular, seem to be also involved in ligand selection in the in vivo interaction of C-type lectins with complex carbohydrate structures. Domains identified by sequence comparison and predicted to have an overall folding similar to that seen in the crystal structure of the C-type lectin domain of MBP (Weis et al., 1991b), but lacking some of the characteristic residues involved in calcium/carbohydrate binding, are seen in a number of type II membrane proteins (Table 1). These exceptions include the mu-

Table 1. *Proteins containing C-type lectin domains or homologous regions*[a]

	Specificity known	Reference	Gal/Man type
1. Proteoglycans			
Rat cartilage proteoglycan core protein	Gal-Fuc	Doege et al., 1987	QPD-E-WND gal
Human cartilage proteoglycan core protein	Gal-Fuc	Doege et al., 1991	QPD-E-WND gal
Chicken cartilage proteoglycan core protein	Gal-Fuc	Tanaka et al., 1988	QPD-E-WND gal
Fibroblast proteoglycan protein	Gal-Fuc	Zimmerman & Ruoslahti, 1989	QPD-E-WND gal
2. Type II membrane proteins			
Chicken hepatic lectin	GlcNAc	Drickamer, 1981	EPN-E-WND man
Human asialoglycoprotein receptors 1 and 2	Gal-GalNAc	Spiess & Lodish, 1985	QPD-E-WND gal
Rat asialoglycoprotein receptor	Gal-GalNAc	Drickamer et al., 1984	QPD-E-WND gal
Rat peritoneal macrophage lectin	Gal-GalNAc	Ii et al., 1990	QPD-E-WND gal
Rat Kupffer cell lectin	Gal-Fuc	Hoyle & Hill, 1991	QPD-E-WND gal
Murine macrophage lectin	Gal-GalNAc	Sato et al., 1992	QPD-E-WND gal
Human placenta-derived gp120 binding lectin	Man	Curtis et al., 1992	EPN-E-WND man
Human CD23	•	Suter et al., 1987	EPN-E-WND
Murine T lymphocyte antigen (A1)	•	Yokoyama et al., 1989	----G-RLD
Murine T lymphocyte antigen (YE1/48)	•	Chan & Takei, 1989	---------
Rat NKR-P1	•	Giorda et al., 1990	----D-VLS
Human Lyb-2	•	Van Hoegen et al., 1990	KPF-D-WIM
Human NKG2 (A–D)	•	Houchins et al., 1991	----L-LKS
Murine NK cell protein Ly49	•	Wong et al., 1991	----G-LDN
Human T cell hybridoma lectin homologue	•	Yoshimatsu et al., 1992	QPV-G-WRR
3. The collectins			
Bovine conglutinin	GlcNAc	Lee et al., 1991	EPN-E-WND man
Human mannan binding protein	Man-Fuc	Taylor et al., 1989	EPN-E-WND man
Rat mannan binding proteins A and C	Man-Fuc	Drickamer et al., 1986	EPN-E-WND man
Human lung surfactant protein A	Man-Fuc	White et al., 1985	EPA-E-WND
Dog lung surfactant protein A	•	Benson et al., 1985	EPR-E-WND
Human lung surfactant protein D	Malt-Man	Rust et al., 1991	EPN-E-WND man
Bovine collectin-43	Man-ManNAc	Holmskov et al., 1993b	EPN-E-WND man
Bovine mannan binding protein	Man-Fuc	Holmskov et al., 1993a	EPN-E-WND man
4. The selectins			
L-selectin	NeuNAc-Fuc	Lasky et al., 1989	EPN-E-WND man
E-selectin	NeuNAc-Fuc	Bevilacqua et al., 1991	EPN-E-WND man
P-selectin	NeuNAc-Fuc	Johnson et al., 1989	EPN-E-WND man
5. Other C-type lectins			
Human HIP	Lac	Christa et al., 1994	EPN-G-WKD
Sea raven antifreeze protein	Ice	Ng & Hew, 1992	KPD-A-WDD
Smelt antifreeze protein	Ice	Ewart et al., 1992	QPD-E-WND gal
Rat pancreatic thread protein	$CaCO_3$	Rouquier et al., 1991	YPN-G-WRD
Macrophage mannose receptor	Man	Taylor et al., 1990	
CRD-1			SPS-K-WEN
CRD-2			EPS-E-WAD
CRD-3			MPG---VDV
CRD-4			EPN-W-WND man
CRD-5			EPN-V-WND man
CRD-6			YPG-A-WMD
CRD-7			EPK---WKT
CRD-8			DPS---WSN

[a] Proteins containing C-type lectin domains or regions with homology to C-type lectin domains are listed and are divided into 5 groups (Drickamer, 1992). Not all of the carbohydrate specificities have been determined (•), and certain proteins, although they contain the conserved residues consistent with a C-type lectin domain being present, do not appear to bind carbohydrate (e.g., the sea raven antifreeze protein). The mannose or galactose types indicate the presence of residues (Drickamer, 1992) shown to correlate with the binding specificity of the particular lectin domain for either galactose or mannose.

rine T lymphocyte antigens A1 (Yokoyama et al., 1989) and YE1/48 (Chan & Takei, 1989), the murine natural killer cell protein Ly49 (Wong et al., 1991), and its rat homologue NKR-P1 (Giorda et al., 1990), as well as the human and murine Lyb-2 proteins (Van Hoegen et al., 1990). Also lacking one of the conserved residues considered to be involved in MBP-carbohydrate interaction is the human IgE receptor CD 23, which binds in a calcium-dependent manner to IgE, but also to nonglycosylated

IgE (Vercelli & Geha, 1989). The macrophage mannose receptor, which is the only protein known to contain within its primary sequence more than a single C-type lectin domain, also falls into this category. Only 2 of the 8 domains show all of the calcium-binding residues required for interacting with a carbohydrate ligand seen in the MBP crystal structure. Interestingly, significant sequence homology to C-type lectin domains is also seen in a group of antifreeze proteins from herring, sea raven, and smelt (Ewart et al., 1992; Ng & Hew, 1992). These polypeptides inhibit the growth of ice crystals in a calcium-dependent manner; however, there is no evidence yet whether the interaction with ice crystals is similar to that of C-type lectins to hydroxyl groups of carbohydrates.

The α-helical bundle

Recently the structural characteristics of the domain located immediately N-terminal to the lectin domain, and which is common to all of the collectins, were established (Hoppe et al., 1994). The short stretch of 34–41 amino acids between the N-terminal end of the lectin domain and the beginning of the collagen-like triple helix contains hydrophobic residues that show a heptad repeat (Fig. 2). A recombinant peptide equivalent to this region in human SP-D was analyzed, by size exclusion chromatography and crosslinking experiments, and found to form a trimer in solution. CD measurements indicated mostly α-helical structure and multidimensional NMR revealed the parallel nonstaggered orientation of the α-helices in a 3-stranded coiled-coil. The structure is held together by very strong, hydrophobic forces and proved to be very stable against denaturation by heat ($T_m > 55$ °C) or pH (pH 3.0–8.5). Coiled-coils are found in a variety of modular proteins, and can contain 2, 3, or 4 α-helices in parallel and nonstaggered, or antiparallel and staggered, orientation. The strong and noncovalent forces that hold these helices together result from hydrophobic side chains (Harbury et al., 1993), in a and d positions of the heptad repeat, forming the interface between the helices (Fig. 2). However, other side chains also play an important role in the interactions between the helices and may be crucial to the interchain recognition process in the folding pathway of these molecules. SP-D, conglutinin, and CL-43 show a good agreement with the heptad repeat pattern, whereas irregularities occur in the sequences of MBP and SP-A (Fig. 2). However, hydrophilic or charged residues in positions a and d can also be found in the chains of other α-helices of known coiled-coil structure, such as laminin (Timpl et al., 1979) and the macrophage scavenger receptors (Kodama et al., 1990).

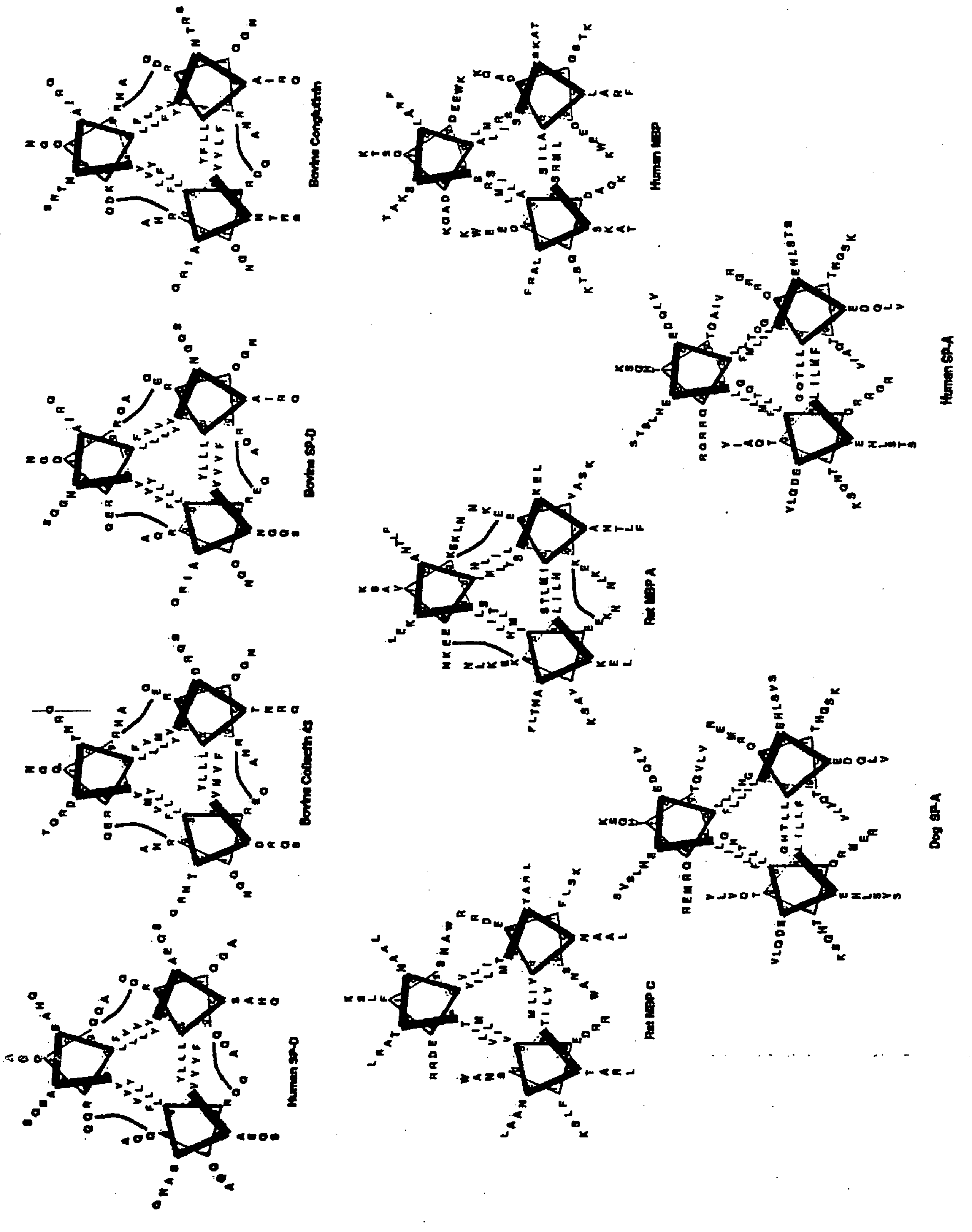
The collagenous domain

The collectins are a family of soluble proteins containing collagenous sequence. Most collagens are insoluble constituents of the extracellular matrix, such as the fibril-associated collagens with interrupted triple-helix (FACITs) (collagen types IX, XII, XIV, and XVI) and the collagens of striated fibrils (types I, II, III, V, and XI) (Van der Rest & Garrone, 1991). C1q is well known as a serum protein containing collagen-like triple helical structures (Reid, 1983), and its C-terminal globular domains show significant similarity to the C-terminal noncollagenous regions of the chains of the ground squirrel hibernation protein (Takamatsu et al., 1993) as well as to the C-terminal regions of the type VIII and X collagens (Sellar et al., 1991), and to the precerebellin molecule (Urade et al., 1991) (Table 2). However, none of these molecules shows a C-type lectin activity or sequence similarity to the lectin domain or appears to contain an α-helical bundle. This also holds for the ficolins (Ichijo et al., 1993), a newly discovered class of molecules bearing fibrinogen-like domains at their C-terminal ends that, like the C1q B chain, conglutinin, and SP-D, contain noninterrupted collagenous regions within the N-terminal halves of their polypeptide chains. The features of the collagenous regions of the collectins and other soluble collagen-containing proteins are compared in Table 2A.

The biosynthesis of collagen-containing molecules requires the correct alignment of 3 polypeptides of repeating Gly-Xaa-Yaa triplets to form the triple-helix (Traub & Piez, 1971). As the formation of the collagen helix proceeds in a zipperlike fashion from a single nucleation point at the C-terminal end of the 3 chains (Engel & Prockop, 1991) and as no direct association between the C-type lectin domains can be observed under physiological conditions (Weis et al., 1991a) it is possible that, in the collectin family of proteins, the tight association of the 3 chains in the α-helical bundle serves as the nucleation point for the formation of the triple-helix (Hoppe et al., 1994). Characteristic side-chain modifications known from collagen molecules are also present in the collagenous portion of the collectins, as for example in the case of MBP, which was shown to carry glycosylgalactosyl modifications on a lysine residue (Colley & Baenzinger, 1987) similar to those seen in other collagens and C1q. Many of the proline and lysine residues in the Yaa-position are hydroxylated to give 4-hydroxyproline and hydroxylysine, respectively. Hydroxyproline residues are known to stabilize the collagen triple-helix (Sakakibara et al., 1973) and presumably carry the same function within the collectins. However, the hydroxylysine residues do not appear to participate in the covalent crosslinking reactions mediated by lysyl oxidases as is seen in the collagens of the extracellular matrix (Eyre et al., 1984). In the collectin family of proteins the association of the trimeric collagen-containing rods to form higher order oligomers appears to be mediated predominantly by the N-terminal noncollagenous sequences and often involves disulfide bridges between the subunits (Lu et al., 1993c). The association between the heterotrimeric subunits in the C1q molecule, as well as in the SP-A molecule, involves almost a third of the collagen triple-helix and is characterized by not only disulfide bonds but predominantly by hydrophobic residues thought to face those of the neighboring triple-helices in the collagenous region up to the bend in the bunch-of-tulips-like structure (Kilchherr et al., 1988). However, in the C1q molecule, a region of charged amino acids, in the Xaa- and Yaa-positions, exhibits a significant degree of homology to the regions in the triple-helical domain following the N-terminal domains of the collectin molecules. This region has been postulated to be involved in the interaction between both C1q and the collectins with the C1q receptor (Malhotra et al., 1993b).

The N-terminal region of the collectins

The N-terminal, noncollagenous, sequences of the collectins show only limited similarity to each other and no homologous regions in other proteins were retrieved from the databank in searches with these sequences. However, the conservation of the cysteine residues between conglutinin, CL-43, MBP, and SP-D


Bovine Conglutinin
Bovine SP-D
Bovine Collectin 43
Human SP-D
Human MBP
Rat MBP A
Rat MBP C
Human SP-A
Dog SP-A

is indicative of a similar role for these residues, in the association to higher oligomeric forms (as illustrated in Fig. 1B).

Bovine conglutinin was found to contain an additional cysteine residue within its collagenous domain. This cysteine residue in the interruption of the triple-helix at triplet 8 has been implicated in the increased stability of the disulfide-linked oligomers seen in the electron micrographs and in SDS-PAGE analysis of conglutinin when compared to bovine SP-D (Lu et al., 1993c). The purification of intact oligomers proved to be more difficult in the case of SP-D than of conglutinin and a large excess of monomers is seen in electron microscopy pictures, depending on the preparation; no higher oligomers have as yet been detected in preparations of the recently discovered CL-43 molecule from bovine serum (Holmskov et al., 1993b).

Function

Carbohydrate recognition by the collectins

An important feature of C-type lectin domains is the very low affinity of a single domain for its ligand. Thus, it is not surprising that C-type lectin domains have mainly been found in molecules containing regions mediating oligomerization or membrane attachment. Native MBP, for example, contains 18 lectin domains and exhibits a K_D for mannan of 2×10^{-9} M (Kawasaki et al., 1983), whereas the binding of single recombinant lectin domains of human SP-D to immobilized carbohydrate ligands on plates or resins can be completely inhibited by 0.1 mM glucose (Hoppe et al., unpubl. obs.). Lectin domains in trimeric forms, such as those found in dissociated single subunits of collectins (Fig. 1A) or in recombinant molecules containing the α-helical bundle of human SP-D, can be bound to immobilized monosaccharides and can only be completely eluted when a concentration of free monosaccharide of 20 mM is used (Hoppe et al., unpubl. obs.). However, the degree of multivalency is only 1 structural factor determining ligand specificity of the collectin molecules.

Structural factors influencing carbohydrate binding appear to operate at 3 distinct levels: at the level of the residues directly involved at the binding sites of both the lectin domains and the carbohydrate; at the level of steric hindrance by either protein or carbohydrate structure restricting access to the respective binding sites; and at the level of epitope spacing on the ligand, which has to match the position of the lectin domains in a given collectin as determined by the overall 3-dimensional assembly. Therefore, the conformation of the neighboring hydroxyl groups at the exposed sites of the carbohydrate has to match the subtype of the C-type lectin (this explains the failure of galactose and the success of L-fucose in inhibiting MBP binding to mannan), and the conformation of the carbohydrate around the hydroxyl groups directly involved in binding has to be sterically favored by other side chains within the lectin domain (e.g., the increasing size of the groups substituted at the 2-hydroxyl group position in glucose reduces the ability of the sugar to inhibit binding of SP-D to maltosyl-BSA [Lu et al., 1993a], with 50% inhibition being shown by 10 mM glucose or by 19 mM glucosamine or by 29 mM *N*-acetyl glucosamine). Also, a suitable binding site must be present within the carbohydrate moiety and must not be masked by other parts of the structure of the glycoproteins or complex carbohydrates (the recognition of the carbohydrate moiety in iC3b but not in the native C3 by conglutinin in the fluid phase is a good example of the biological significance of this level of regulation of specificity [Lachmann & Müller-Eberhard, 1968]).

However, the unusual overall structures of the collectin molecules may very well have an additional specificity-determining influence in the in vivo situation; for example, conglutinin spans almost 100 nm with its collagenous regions compared to a maximal distance of 28 nm between the lectin domains in the MBP molecule (Strang et al., 1986) (Fig. 1B). It seems unlikely, therefore, that both of these serum collectins exhibit identical binding to the carbohydrate moieties on target particles of different size and shape, such as influenza virus (100 nm), poliomyelitis virus (30 nm), or *Escherichia coli* (1,000 nm), even if those particles contain accessible carbohydrate molecules with the required OH configuration in a comparable density on their surface. The requirement for multiple binding sites on the same particle becomes especially relevant in the case of lectin–ligand interaction where only a single lectin domain, or a lectin "head" composed of a trimeric group of lectin domains, is involved in binding, such as would be required for the crosslinking by collectin molecules of small nonaggregated ligands, e.g., soluble asialoglycoproteins. These interactions should be readily inhibitable by normal serum concentrations of glucose, and this thus represents another factor determining ligand specificity in the in vivo situation. Truncated conglutinin is the major form of conglutinin in heat-inactivated bovine serum and lacks the N-terminal 40 residues, resembling a trimeric molecule containing a collagenous triple-helix and a single lectin "head," and cannot form higher oligomers. This truncated conglutinin molecule was shown to exhibit neither conglutination activity nor binding activity to the sensitized erythrocyte-solid phase iC3b complex (EAiC3b), but it retained the binding activity to mannan in solution and the original binding specificity for *N*-acetylglucosamine (Kawasaki et al., 1983; Lu et al., 1993c). The different shapes of the collectins as well as the length of the collagenous region and their oligomeric assembly all seem capable of influencing the binding specificity for carbohydrate ligands on biologically relevant particles, yet comparative studies identifying distinct targets for the binding of different collectins on an appropriate target

Fig. 2. Wheel projection of the amino acids forming the α-helical bundle. The structure of the α-helical bundle present in human SP-D has been determined by multidimensional NMR, and the equivalent regions of the other collectins are shown for comparison. The sequences begin at the N-terminal end of the α-helical region, with hydrophobic residues shown in green, positively charged residues shown in red, and negatively charged residues shown in blue. The brown line indicates a possible interaction between residues in e and g positions. The wheel projections for bovine SP-D, conglutinin, and collectin-43 are in excellent agreement with the observed structure of human SP-D and illustrate the strong hydrophobic interactions between the helices. The wheels representing MBP and SP-A show a number of hydrophobic residues in positions b, c, e, f, and g, pointing "outward," and also contain hydrophilic residues in the buried positions a and d. The model drawing of these 2 collectins therefore indicates that the forces holding the α-helical bundle together might not be as strong as those seen in the human SP-D molecule.

Table 2. *Comparison of soluble proteins containing collagenous regions*

A. Soluble proteins containing collagenous regions[a]

Protein, origin	Leader length (a.a.)	N-terminal region: Length (a.a.)	N-terminal region: Cysteines	Collagen region: Length (a.a.)	Collagen region: Cysteines	Collagen region: Interrupted triplet (no.)	Collagen region: Length (EM or calc.)	Neck region length (a.a.)	Globular domain: Length (a.a.)	Globular domain: Domain type
SP-D, human	20	25	2	177	–	–		38	115	C-type lectin
SP-D, rat	19	25	2	177	–	–		38	115	C-type lectin
SP-D, bovine	20	25	2	171	–	–	46 nm	38	115	C-type lectin
Conglutinin, bovine	20	25	2	171	1	GRECPH (4)	39 nm	38	117	C-type lectin
Collectin-43, bovine	20	28	2	114	–	–	37.4 nm	41	118	C-type lectin
SP-A, human	17	10	1	71	–	GEMPCPP (13)	10 + 10 nm	34	114	C-type lectin
MPB, human	21	21	3	59	–	GGTGQ (8)	Ca. 13.2 nm	34	113	C-type lectin
Ficolin α	29	19	1	54	1	–	16 nm (calc.)	NK	220	Fibrinogen
Ficolin β	32	19	1	57	1	–		NK	217	Fibrinogen
HP-20	23	1	0	39	–	–	11 nm (calc.)	NK	133	Clq-like
HP-25	28	11	1	42	–	–		NK	135	Clq-like
HP-27	30	12	1	39	–	–		NK	134	Clq-like
Clq A	22	8	1	79	–	GIRT (10)	11 + 12 nm	NK	136	Clq
Clq B	27	11	1	78	–	–		NK	136	Clq
Clq C	28	5	1	81	–	GIPAIK (11)		NK	131	Clq

B. Cysteines in the N-terminal region[b]

Protein	Sequence	Origin
SP-D	AEMTTFSQKILANACTLVMCSPPEDGLPG	Bovine
SP-D	AENKTYSHRTTPSACTLVMCSSVESGLPG	Human
SP-D	AENKTLSQRSITNTCTLVLCSPTENGLPG	Rat
Conglutinin	AEMTTFSQKILANACTLVMCSPLESGLPG	Bovine
Collectin-43	EEMDVYWEKTLTDPCTLVVCAPPADSLRG	Bovine
MBP	ADTETETNEENIRKTCPVIACGPGING	Bovine
MBP	ETVTCEDAQKTCPAVIACSSPGING	Human
MBPc	AVYAETLTEGAQSSCPVIACSSPAWNG	Rat
MBPc	ETLTEGAQSSCPVIACSSPGLNG	Mouse
MBPa	VSSSGSQTCEETLKTCSVIACGRDGRDGPK	Rat
MBPa	SGSQTCEDTLKTCSVIAC	Mouse
SP-A	EVKDVCVGSPGIPGTP	Human
SP-A	VSGIENNTKDVCVGNPGIPGTP	Dog
Clq A	MVTEDLCRAPDGKKGEAGRPGRR	Human
Clq B	GLIDISQAQLSCTGPPAIPGIPGIPGTP	Human
Clq C	ALRGQANTGCYGIPGMPGLPGAPGKD	Human

[a] Domain organization of the collectins and comparison of their sequences with those of the ficolin molecules, the 3 chains of Clq, and the hibernation protein. a.a., Amino acids; EM, determined by electron microscopy; NK, not known.
[b] Conserved cysteine residues present in the N-terminal regions of the collectins and comparison of these regions with the N-terminal sequences of the 3 chains of Clq.

microorganism, such as *Salmonella* spp., have not yet been published.

As the activation of the C1 molecule requires only 2 immobilized IgG molecules to interact with 2 (heterotrimeric) globular "heads" of the Clq molecule (Kilchherr et al., 1986), it seems reasonable to assume a similar situation for the interaction of the serum MBP-MASP (MASP = MBP-associated serine protease) complex (see below) with activating carbohydrate-bearing particles (Matsushita & Fujita, 1992). Complement-related, functionally relevant binding levels of MBP to surface carbohydrate structures might thus be considerably lower than those required for the opsonizing and phagocytosis-enhancing functions common to MBP and other collectins via their interaction with the Clq receptor, present on a variety of cells. A single molecule of MBP can activate the complement system and thereby cause the deposition of iC3b molecules on the activating surface, whereas much higher numbers of MBP molecules have to be bound to a surface of a particle to trigger the phagocytosis-enhancing functions involving the Clq receptor.

Binding of the collectins to bacteria and viruses

MBP is part of the complement-dependent RaRF bactericidal factor that is present in the sera of a wide range of vertebrates (Kawakami et al., 1984; Ji et al., 1993). This factor is composed of the MBP plus the enzyme MASP. MASP, like C1s, can activate components C4 and C2 of the classical pathway of com-

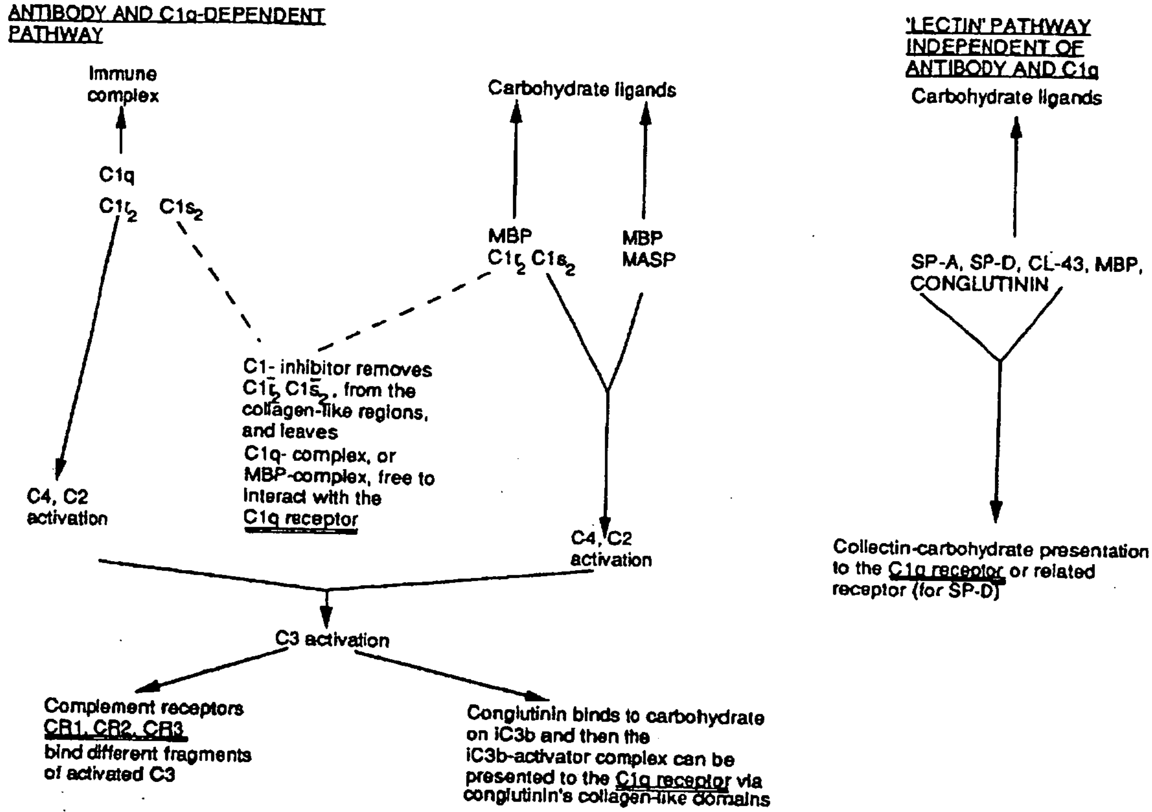


Fig. 3. Interaction of C1q and the collectins with the complement system. Activation of the classical pathway of complement via C1q binding to immune complexes or via the collectins binding to carbohydrate ligands. MASP is considered to be the protease associated with MBP in physiological conditions; however, it is not yet known if MASP associates with the collagen-like region of MBP. It is not known if MASP is bound and inactivated by the serum C1-inhibitor.

plement (Ji et al., 1993) (Fig. 3). The RaRF factor was first isolated by virtue of its calcium-dependent lectin binding to the rough Ra-chemotype strains of *Salmonella* and the rough R2 strains of *E. coli.* Enhanced phagocytosis of MBP-coated *Salmonella montevideo* is shown by human monocytes (Kulhman et al., 1989), but this appears to be due to a direct interaction of MBP with monocyte receptors rather than by means of complement activation and complement receptors. MBP interacts with envelope glycoproteins of HIV-1 and HIV-2 and, in serum, this results in activation of the classical pathway (Haurum et al., 1993), which could lead to lysis and/or opsonizing of the virus. On the other hand, inhibition of infection of a T-cell line by HIV-1 was achieved by prior incubation of HIV-1 with MBP, in the absence of complement (Ezekowitz et al., 1989). Bovine serum conglutinin has been shown, in vitro, to have antibacterial activity against *Salmonella typhimurium* and *E. coli*, with the activity requiring the presence of an intact complement system and macrophages (Friis-Christiansen et al., 1990), thus suggesting that the conglutinin was playing a crosslinking role between the iC3b-coated microorganisms and complement/collectin receptors. Bovine conglutinin binds, in a calcium-dependent and lectin fashion, to the recombinant form of the gp160 envelope glycoprotein of HIV-1 and inhibits the binding of the gp160 to the CD4 receptor on CEM 13 cells (Andersen et al., 1990). Recently, Ushijima et al. (1992) have purified a human conglutinin-like protein that bound to gp120 of HIV-1 and prevented infection of CD4 positive a CEM lymphoblastoid cell line by HIV-1. Conglutinin and MBP also appear likely to be the β-inhibitors present in bovine serum that have been reported to inhibit the infectivity and hemagglutinating activity of different influenza viruses (Andersen et al., 1990). The ability of bovine serum CL-43 to bind to microorganisms has not yet been reported.

SP-A is a major protein in the pulmonary surfactant, which is a complex of lipids and proteins that form an interface between the aqueous film on the alveolar epithelium surface and the air in the alveolar space (Weaver & Whitsett, 1991). SP-A appears to enhance the activities of the hydrophobic surfactant proteins SP-B and SP-C and it may also be involved in the recycling of surfactant and inhibition of surfactant secretion. However, in addition to these properties, SP-A also has been shown to enhance the phagocytosis of bacteria and viruses by alveolar macrophages (Van Iwarden et al., 1991) and to induce the production of oxygen radicals by these cells. The binding of SP-A, via its lectin domain, has been shown to take place with the gp120 glycoprotein of *Pneumocystis carinii* (Zimmerman et al., 1992) and with *Staphylococcus aureus* (McNeely & Coon-

rod, 1993), the latter binding enhancing the adherence of the SP-A coated bacteria to alveolar macrophages. SP-D is present at an approximately 10-fold lower level in the lung than SP-A, as judged by analysis of bronchoalveolar lavage. SP-D, unlike SP-A, does not show a strong affinity for phospholipids and therefore it is found in the lipid-free portion of bronchoalveolar lavage fluid. SP-A is perhaps a stronger candidate for playing a role in antibody-independent defense against lung infections or in the removal or presentation of allergens. For example, SP-D can bind to, and agglutinate, *E. coli*, probably via carbohydrate structures within the surface lipopolysaccharides (Kuan et al., 1992), which is strongly suggestive that SP-D plays a role in the lung's defence against Gram-negative bacteria.

Interaction of the collectins with the complement system and the C1q/collectin receptor

C1q, along with the calcium-dependent proenzyme, $C1r_2C1s_2$ complex, forms C1, the first component of the classical pathway of complement. The C1 component is activated upon its interaction with immune complexes by the binding of the globular heads of C1q to specific sites on the C_H2 domains of antibody IgG, or the C_H3 domains of antibody IgM. This binding of C1q, to the Fc regions of antibodies, results in activation of both C1r and C1s and the initiation of the complement cascade via activation of both C4 and C2 by C1s. The $C1r_2C1s_2$ complex is known to be bound to the collagen-like regions of C1q and, after activation, to be rapidly removed and inactivated by the control protein C1-inhibitor (Reid, 1983). This leaves the collagen-like regions of C1q free to interact with the C1q receptor (which is known to also interact with many of the collectins). In view of the striking structural similarity between C1q and the collectins (especially SP-A and MBP), it is perhaps not too surprising that the collectins show certain functional similarities to C1q (Holmskov et al., 1994). For example, it is now clear that MBP can mimic the action of C1q in bringing about the activation of the classical pathway of complement but it appears that it is the only collectin that has this property. On the other hand, all the collectins, with the probable exception of SP-D, are bound by the C1q receptor.

MBP, after binding to carbohydrate ligands, has been shown to activate the proenzyme $C1r_2C1s_2$ complex (Lu et al., 1990; Ohta et al., 1990) and thus bring about activation of components C4 and C2 of the classical pathway of complement. However, it seems likely that in plasma MBP is normally complexed with the proenzyme MASP and that, upon binding of the MBP-MASP complex to suitable ligands, the MASP is activated and it is this enzyme that normally brings about complement activation mediated via MBP (Matsushita & Fujita, 1992; Ji et al., 1993; Takada et al., 1993). Although it has been shown that the 80-kDa MASP enzyme is about 36% identical in amino acid sequence to both C1r and C1s, and has the same overall domain organization, it is still not known with certainty what the stoichiometry is with respect to MBP and MASP in the MBP-MASP complex, although the most recent studies suggest a ratio of 1:1 (Ji et al., 1993). Also it is not clear if the MASP is bound to the collagen-like regions of MBP or if the activated MASP is, like activated C1r and C1s, controlled by the C1 inhibitor. As discussed below, the MBP-MASP complex thus provides a route for activation of the classical pathway of complement that is triggered by carbohydrate ligands and is independent of both antibody and the C1 complex.

Conglutinin also exhibits leucocyte- and complement-dependent bactericidal activity but in contrast to MBP this does not appear to involve a direct activation of complement by the carbohydrate ligand-conglutinin complex. The opsonic effects seen with conglutinin are dependent upon prior activation of complement taking place up to at least the C3b stage so that the bacterial target becomes coated with large numbers of C3b molecules. The C3b is converted to iC3b by the enzyme factor I, in the presence of the cofactor H, and this exposes a carbohydrate structure on the α' chain of iC3b, which is recognized and bound by conglutinin. It seems likely that some of the opsonic effects and triggering of other effector functions are mediated by the interaction of the collagen-like regions of conglutinin with the C1q/collectin receptor as outlined below.

MBP, conglutinin, CL-43, and SP-A all appear to bind to the cell-surface C1q receptor, which is consequently also referred to as the "collectin receptor" (Malhotra et al., 1992). The C1q receptor is found on a wide variety of cell types, including most leukocytes, endothelial cells, eosinophils, platelets, fibroblasts, and neutrophils. A number of C1q-mediated cellular responses have been described, including the enhancement of monocyte phagocytic activity, stimulation of fibroblast adhesion, stimulation of an oxidative burst in neutrophils, enhancement of phagocytosis by pulmonary endothelial cells, inhibition of collagen-induced platelet aggregation, and serotonin release (Tenner, 1993). If these, and other, responses attributed to triggering of the C1q receptor by C1q are also set in motion by binding of complexes, composed of collectins bound to carbohydrate ligands on the surfaces of microorganisms, then a wide variety of cellular responses in the blood and lung fluids might be expected as a result of collectins interacting with the C1q receptor. The C1q receptor is an acidic protein, which behaves as an elongated molecule of 115 kDa after purification using detergents (Malhotra et al., 1993c). The detergent-solubilized molecule is composed of 2 identical chains, each of approximately 60 kDa, which show a remarkable degree of sequence identity to the intracellular, calcium-binding, protein calreticulin. However, it is considered, in view of certain differences in the structural and antigenic properties between the 2 proteins, that although they are very similar they may not be identical (Malhotra et al., 1993c). The binding of C1q to its receptor, and the collectins known to bind the C1q receptor, is considered to take place via their collagen-like regions in a Ca^{2+}-independent manner. The collagen-like regions of C1q are masked by the $C1r_2C1s_2$ proenzyme complex and upon activation the activated C1r and C1s are rapidly removed by the C1-inhibitor thus exposing the collagen-like regions and allowing the antibody–antigen–C1q complex to be presented to the C1q receptor. A similar situation may apply to MBP due to its association in plasma with the proenzyme form of the MASP protease. Thus, it is likely that upon activation the MASP enzyme is removed, allowing the carbohydrate ligand–MBP complex to be presented to the C1q receptor via the collagen-like regions in MBP. Studies on the binding of intact and truncated conglutinin to the C1q receptor indicate that the receptor binding site in conglutinin lies within the first 54 N-terminal amino acid residues in the molecule (Malhotra et al., 1993b). Comparison of the N-terminal regions of the chains of C1q, MBP, conglutinin, CL-43, and SP-A has led to the identification of a possible receptor bind-

ing site, composed of 5 Gly-Xaa-Yaa triplets with many charged residues in the X and Y positions, within the N-terminal portions of the collagenous regions of all these molecules (Malhotra et al., 1993b). However, this site is also present in SP-D, which does not appear to bind to the C1q receptor but to bind to a different, but probably related, receptor on alveolar macrophages (Miyamura et al., 1994).

SP-A has been found to bind to pollen grains, and also to specific proteins in an aqueous extract of pollen grains, in a calcium-dependent and mannose-type fashion (Malhotra et al., 1993a). The interaction between SP-A and the pollen grains/extract allows the presentation and adhesion of the pollen grains/extract to A549 alveolar type II cells to take place, probably via the collagen-like regions in SP-A binding to the C1q/collectin receptor, thus suggesting that this receptor may play a role in removal of potential allergens.

Biosynthesis, tissue location, and genetics

cDNA and genomic cloning of the collectins

The liver appears to be the major site of synthesis of both the tissue and serum forms of MBP, since this collectin was first characterized from rabbit, rat, and human liver (Kawasaki et al., 1978; Wild et al., 1983) and subsequently, in higher oligomeric forms, from the sera of these and other species. MBP has also been shown to be synthesized by hepatocytes and liver cell lines. As judged by cDNA, and genomic cloning studies, there are 2 forms of rat and mouse MBP (the A and C forms), which show 55% identity in amino acid sequence within species (Drickamer et al., 1986; Sastry et al., 1991; Wada et al., 1992). Only 1 sequence for human MBP has been described so far, by cDNA and genomic cloning, and it shows approximately 62% identity in amino acid sequence to both the A and C forms of rat MBP (Ezekowitz et al., 1988; Sastry et al., 1989; Taylor et al., 1989). The human MBP gene (Fig. 4) is 7 kb long and contains 4 exons: exon 1 encodes the leader peptide, N-terminal region and part of the collagen-like region; exon 2 encodes the remainder of the collagen-like region; and exon 3 encodes the sequences that form the short α-helical bundle located just prior to the C-terminal C-type lectin domain, which is encoded by exon 4.

SP-A is synthesized in the lung by the alveolar type II cells and, as judged by cDNA and genomic cloning, there appear to be at least 2 expressed human SP-A genes (SP-A I and SP-A II; White et al., 1985; Floros et al., 1986; Katyal et al., 1992). The human SP-A structural gene (gene symbol SFTP 1) is 4.5 kb long and contains 5 exons, with exon 2 encoding the signal peptide, the amino-terminal region, and the first 10 Gly-Xaa-Yaa triplets, and exon 3 encoding the remaining 13 Gly-Xaa-Yaa triplets. Thus, as in MBP, C1q, and type IV collagen, an intron is found within the codon for the glycine located between 2 and 9 residues before an interruption to the repeating nature of the Gly-Xaa-Yaa sequence (since in SP-A the repeats are interrupted after the 13th triplet by the sequence Pro-Cys-Pro-Pro). Exon 4 and part of exon 5 encode a putative amphipathic α-helical region, whereas the remainder of exon 5 encodes the C-type lectin domain. The 2 SP-A genes that are expressed encode for polypeptides differing at only 6 amino acid positions (White et al., 1985; Katyal et al., 1992). SP-A has been cloned at the cDNA level in a variety of other species, such as dog, rabbit, rat, and mouse (Korfhagen et al., 1991). So far, only single SP-A genes have been reported to be present in the rat, rabbit, and mouse.

SP-D is synthesized by alveolar type II cells and has also been reported to be present in alveolar macrophages (in endocytic and lysosomal structures) and in the secretory granules of bronchiolar "Clara" cells. Human SP-D has been cloned at the cDNA (Rust et al., 1991; Lu et al., 1992) and genomic (Crouch et al., 1993) levels. The human SP-D gene (gene symbol SFTP 4) is approximately 11 kb long and contains 8 exons (Fig. 4): exon 2 encodes the signal peptide, the N-terminal region, and the first 7 Gly-Xaa-Yaa triplets; exons 3–6 are all 117 bp in length and encode the remainder of the collagen-like region; and exon 7 encodes the sequences that form the α-helical bundle that links the collagenous regions to the C-type lectin carbohydrate recognition domain (which is encoded by exon 8). SP-D has been cloned at the cDNA level in rat and bovine systems, but no data are yet available at the gene level in these species.

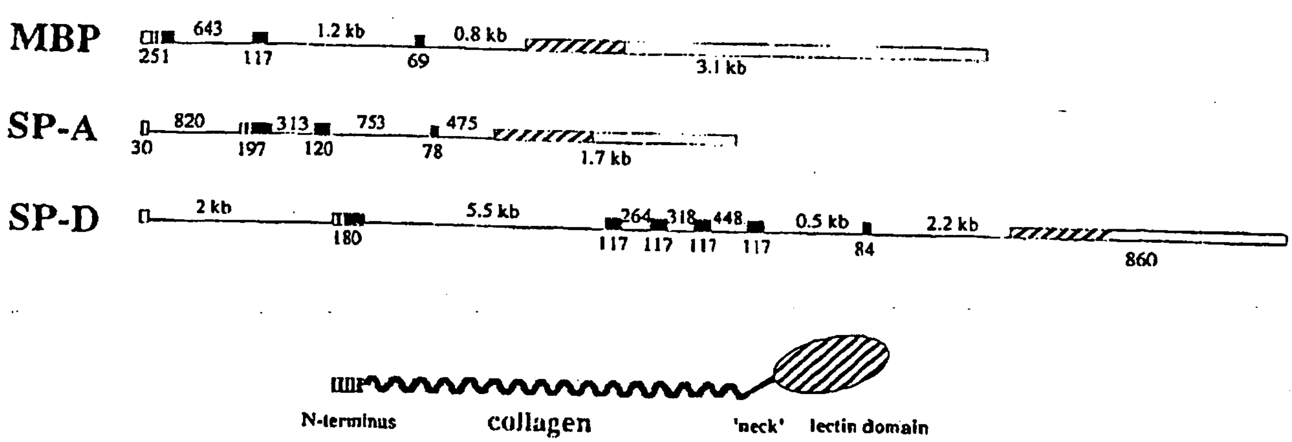


Fig. 4. Organization of the genes of MBP, SP-A, and SP-D on the genomic level. The genes for SP-A, SP-D, and MBP have been mapped to chromosome 10 in humans. Four exons were found to encode MBP, whereas the genes encoding the surfactant proteins A and D span 5 and 8 exons, respectively.

Cluster of genes encoding collectins on human chromosome 10

The genes for MBP, SP-A, and SP-D have all been mapped to the long arm of human chromosome 10. The human MBP gene (gene symbol -MBL) has been localized to 10q11.2–q21 (Sastry et al., 1989), and the human SP-A gene(s) (gene symbol SFTP 1) have been localized to 10q21–q24 (Fisher et al., 1987). There appear to be at least 2 SP-A genes (SP-AI and SP-AII; White et al., 1985; Floros et al., 1986; Katyal et al., 1992) and 1 SP-A pseudogene (Korfhagen et al., 1991) forming a closely linked cluster. STS mapping has also allowed localization of the human SP-D gene (gene symbol SFTP 4) to 10q23–q23, close to the SP-A genes and a 430-kb genomic Mlu1 fragment has been identified that contains both the SP-D gene and the SP-A gene cluster (Kölble et al., 1993). The presence of the gene for the α 1 chain of human collagen XIII (gene symbol COL 13A1) at 10q 22 is of some interest because this non-fibril-forming collagen has 4 noncollagenous regions of unknown function and therefore the presence of this gene along with the genes for the collectins suggests that an ancestor for a superfamily of genes containing collagen-like regions and carbohydrate recognition domains may have originated on the long arm of human chromosome 10.

Susceptibility to disease associated with genetic deficiency of MBP

It has proved difficult to obtain reliable measurements of SP-A and SP-D levels in bronchoalveolar lavage samples from healthy individuals, or patients, and measurements of conglutinin and CL-43 serum levels have, to date, only been made in cows. However, the serum levels of human MBP in Caucasians are well studied and have been found to vary quite considerably (0–5 μg/mL with an average value of 1.23 μg/mL) with quite a high percentage of individuals showing close to zero and therefore suggesting at least a partial deficiency of MBP.

It has been established that, in humans, a low serum concentration of MBP correlates with a common opsonic defect (Super et al., 1989) and that deficiency of MBP is associated with recurrent infections in the very young (Turner, 1991). It is considered that low serum levels or lack of MBP results in an inability to activate the classical pathway of complement (via MASP or $C1r_2C1s_2$), by an antibody-independent route, and this prevents efficient coating of pathogenic organisms with the activated C4 and C3 fragments that are required for efficient opsonization by cells carrying C4b and/or C3b receptors.

Four allelic forms of MBP have been described: the normal, A, allele; the B allele, which shows a single nucleotide substitution within codon 54 in exon 1, which results in the replacement of a glycine by aspartic acid within the collagen-like region (Sumiya et al., 1991); the C allele, which also shows a single nucleotide substitution, but within codon 57 in exon 1, which results in the replacement of a glycine by glutamic acid (Lipscombe et al., 1992) within the collagen-like region; and the D allele, another point mutation, but within codon 52 of exon 1, resulting in the replacement of an arginine by a cysteine, again within the collagen-like region (Madsen et al., 1994). The substitution of glycine in alleles B and C by charged residues results in disruption of the Gly-Xaa-Yaa repeating structure, and this is considered to prevent the correct formation of a triple-helical structure containing 1 or more of the abnormal alleles. Although the amino acid substitution in the D allele is also within the collagen-like region, it does not disrupt the continuity of the Gly-Xaa-Yaa repeating structure. However, the introduction of a cysteine residue could also cause structural problems due to possible aberrant disulfide bond formation. It is clear that individuals who are homozygous with respect to any of the B, C, or D alleles have undetectable, or trace, amounts of MBP antigenic activity in their sera as judged by sensitive immunochemical assays. The allele frequencies seen in Caucasians for A:B:C:D are 0.81:0.13:0.02:0.05. People who are heterozygous for the normal allele and one of the variant alleles (e.g., A/B heterozygous individuals) do have MBP in their sera that has the same biochemical and functional characteristics as MBP isolated from A/A homozygous individuals. Thus, it is generally agreed that the presence of any of the variant alleles, along with the A allele, decreases the serum concentration of MBP to about 15% of that expected in A/A homozygous individuals. This is consistent with the view that in heterozygous individuals only 1 in any 8 of the MBP "structural units" (composed of 2 triple-helices of disulfide-bonded AAA chains) would be of the normal type while the remaining 7/8 would be abnormal (containing at least one of the variant chains) and probably susceptible to rapid degradation (Sumiya et al., 1991). The fact that the point mutations giving rise to the different allelic forms are clustered within codons 52 and 57 found in exon 1 of the MBP gene is quite striking and indicates that the correct folding of the N-terminal section of the collagen-like region is critical for the stability of MBP.

The identification of the B, C, and D alleles of MBP does not, however, explain the very wide variation seen in serum MBP levels within apparently healthy A/A genotype individuals (0.13–5.0 μg/mL). It is possible that other variants have yet to be identified. Such variants need not necessarily influence the structure of the MBP, but may be affected in the area of the gene responsible for regulation of the level of biosynthesis of MBP.

Conglutinin has, to date, only been fully characterized in the bovine system. Hepatocytes seem likely to be the major site of synthesis, as judged by both immunohistochemical staining (Holmskov et al., 1992) and Northern blotting (Lu et al., 1993b). Bovine conglutinin has been cloned at the cDNA (Lu et al., 1993b) and genomic (Liou et al., 1994) levels. The intron/exon organization of the bovine gene (Liou et al., 1994) is very similar to that of the human SP-D gene, reflecting the high degree of amino acid sequence similarity (86% identity) seen between bovine SP-D and bovine conglutinin (Lim et al., 1993). Although a human lectin with similar structure and functional properties to conglutinin has been described (Thiel et al., 1987; Ushijima et al., 1992), it is still not clear if conglutinin is found in species other than the Bovidae.

Collectin-43, like conglutinin, has only been characterized in the bovine system, where, as judged by Northern blot analysis, it is synthesized in the liver (Lim et al., 1994). It has been cloned at the cDNA (Lim et al., 1994), but not the genomic, level.

Perspectives

The exact compositions of naturally occurring oligosaccharide ligands, for any of the collectins, have not yet been fully identified. The overlapping monosaccharide specificities (Table 3)

Table 3. *Comparative functional activities of the collectins*[a]

Collectin	Principal carbohydrate specificities	C1q receptor binding	Complement activation	iC3b binding	Organisms bound
Human					
MBP	GlcNAc > Man	Yes	Yes	No	*S. montevideo*, *S. typhimurium*, *E. coli*, HIV
SP-A	ManNAc > Fuc	Yes	No	No	*P. carinii*, pollen extract, *S. aureus*, herpes simplex virus
SP-D	Mal > Gluc	No (SP-D receptor?)	No	No	*E. coli*
Bovine					
MBP	Fuc > GlcNAc	Yes	Yes	No	Influenza virus
Conglutinin	GlcNAc > Gluc	Yes	No	Yes	Influenza virus
Collectin-43	Man > ManNAc	Yes	No	No	Not determined

[a] The carbohydrate-binding properties of all collectins are of the mannose type and the monosaccharide specificities indicated; although informative in their own right, they do not allow for speculation about the natural ligands for the collectins (Lu et al., 1992; Haurum et al., 1993; Holmskov et al., 1993a, 1993b). The organisms listed do not reflect comparative studies with more than 1 collectin (Ezekowitz et al., 1989; Andersen et al., 1990; Friis-Christiansen et al., 1990; Van Iwarden et al., 1991; Kuan et al., 1992; Zimmerman et al., 1992; Haurum et al., 1993; McNeely & Coonrod, 1993).

for all of the collectins suggest that the various native oligosaccharide ligands for these molecules may be found on the surface of quite a wide range of microorganisms and target particles. In addition to this, oligosaccharides of known composition can assume in solution a range of conformations, of which even energetically unfavorable structures were shown to act as ligands for lectin binding (Imberty et al., 1993), which is reflected in the lack of success in carbohydrate structure determination using X-ray diffraction. The flexible nature of oligosaccharides clearly makes structural analysis by NMR a more suitable approach to determine lectin–carbohydrate interactions. The occurrence of unordered side chains within close proximity to the binding site of MBP seen in the crystal structure also allows for speculation on there being some degree of flexibility of the protein structure, especially within the extended loop of nonregular secondary structure. Obviously, the determination of the monosaccharide specificities is only a step in the right direction towards fully understanding the interaction between collectins and their biologically relevant targets. The well-defined groups of oligosaccharide ligands, like the sialyl Lewis x family of carbohydrate structures (Tyrrell et al., 1991), were available in the study of selectin mediated cell adhesion, but no such well-defined groups of oligosaccharide ligands have yet been identified to allow study of collectin function. It is important to characterize the natural oligosaccharide ligands for the collectins to allow assessment of collectin-mediated reactions, such as complement activation or opsonization. The determination of ligand specificity of the collectins, beyond monosaccharide recognition, should therefore be a promising future direction of investigation.

All the collectins are soluble molecules and are readily secreted in mammalian expression systems, thus avoiding the use of fusion proteins, which is necessary for producing soluble selectins (Foxall et al., 1992). Collectins also have a constant and defined degree of oligomerization and well characterized overall structures. Study of normal and mutated recombinant molecules should allow the establishment of binding sites for carbohydrates, proteins, or even lipids. The interaction with the complement system and host cell receptors via the collagenous/N-terminal regions of the collectins might allow for the classification of microorganisms according to their ability to be recognized by different collectins. The collectin–microorganism interaction could direct the innate immune response toward either complement activation, opsonization, phagocytosis via the C1q receptor, or utilization of a recently postulated distinct receptor for SP-D (Miyamura et al., 1994). A combination of these reactions may take place, depending on the collectin in question, and the state of inflammation already in progress, especially, since MBP, for example, behaves as an actue phase reactant. The very specific recognition of the high mannose structure in iC3b, but not C3, by bovine conglutinin is a further interaction of this collectin with the complement system. Recent evidence also suggests that allergic reactions to carbohydrate-bearing particles released from pollen grains, or house dust mites, may be affected by the binding of SP-A (Malhotra et al., 1993a) or SP-D to the carbohydrate on the allergen.

As more data become available on proteins showing homology with the C-type lectin domains, it should be borne in mind that not all of the domains identified by sequence alignment as "C-type lectin" domains necessarily bind carbohydrate structures. Some of these proteins have been shown to bind to structures other than carbohydrates and lack some (if not all) of the residues shown in the C-type lectin domains to be responsible for interaction with the calcium ions as well as the carbohydrate molecules, while retaining most of the hydrophobic residues of the core structure, including the disulfide pattern. The pancreatic thread protein binds to $CaCO_3$ crystals, and binding to ice crystals is seen in the case of antifreeze proteins of smelt (calcium-dependent) and sea raven (no calcium requirement) protein. It might therefore be necessary to consider also studying noncarbohydrate ligands on target organisms and particles, especially in the case of SP-A, which lacks one of the residues thought to be involved in ligand binding.

In view of the possible medical importance of collectins in the lung and plasma, the definition of the natural oligosaccharide structures recognized by the collectins, the further characterization of receptors for collectins, and the precise identification of carbohydrate/glycoprotein structures on the surface of microorganisms that are important in collectin-mediated immune re-

actions seem likely to be the dominant lines of investigation in this area in the near future.

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